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STUDIES ON THE NUTRITION OF *PASTEURELLA PESTIS*, AND FACTORS AFFECTING THE GROWTH OF ISOLATED CELLS ON AN AGAR SURFACE.

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THE work to be described in this paper arose out of an attempt to develop an accurate viable count procedure for *Pasteurella pestis*. It was desired to adopt the technique of Miles and Misra (1938), in which standard drops of a suitably diluted suspension are delivered on to the surface of agar plates, which are subsequently incubated and counted. A medium and growth conditions were required which allowed every isolated organism on an agar surface to develop into a colony ; for *Past. pestis* these requirements are not satisfied by ordinary media.

Schütze and Hassanein (1934), in a study of the same problem, found that heavy inocula of *Past. pestis* would grow on the surface of ordinary agar plates, but widely separated single cells would not grow. Growth of single cells could be obtained by adding to the agar either whole blood, pepsinized blood, or extracts of various bacteria. Alternatively, isolated cells would develop on ordinary agar if the plates were incubated anaerobically.

These findings were confirmed, and attempts were made to discover the nature of the substance present in blood that is responsible for this effect, and why it is only required under aerobic conditions. It was also necessary to study the nutrition of *Past. pestis* and its growth in chemically defined media. While the results of this investigation are by no means complete, they have led to an accurate counting technique for *Past. pestis*, and are also thought to be of sufficient interest on their own account for publication at the present time.

METHODS.

Agar media.—The following were used as basal media for agar plates : (1) 2 per cent. tryptic digest of casein containing 1 per cent. sodium glycerophosphate as buffer ; (2) tryptic digest of beef muscle ; (3) 2 per cent Evans' peptone ; all at pH 7·6 and containing 1·5 per cent agar. (2) and (3) gave rather better growth than (1). Peptic digests of sheep's blood were prepared according to Fildes (1920), and as a rule added to the melted agar immediately before pouring plates.

Amino-acid medium.—The medium contained 20 amino-acids in the following concentrations : dl-glutamic acid, M/200 ; glycine, M/500 ; dl-alanine, dl-valine,

dl-leucine, dl-isoleucine, dl-norleucine, dl-aspartic acid, dl-lysine, l-arginine, l-histidine, dl-proline, l-hydroxy-proline, dl-phenylalanine, dl-threonine, all M/1000; dl-serine, dl-tyrosine, dl-cystine, dl-methionine, all M/2000; dl-tryptophane, M/5000. (The above concentrations refer to the final concentration of l-isomer in the medium.) All dl-compounds were synthetic. The medium also contained KH_2PO_4, M/30; $(NH_4)_2SO_4$, M/100; $MgSO_4$, M/5000; $CaCl_2$, M/100,000; $FeSO_4$, M/200,000; $MnSO_4$, $ZnSO_4$, $CuSO_4$, all M/500,000; glucose, M/100, and NaOH to adjust to pH 7·6. The medium was made up and tubed at one and a half times strength, being diluted by the inoculum, other additions and water to make up volume. Five ml. quantities were incubated in 50 ml. Erlenmeyer flasks, in jars containing air/5 per cent CO_2, or H_2/5 per cent CO_2 for anaerobic experiments.

This medium contains most amino-acids in rather higher concentrations than are usual in synthetic media; this was found to give heavier growth.

Viable counts.—Seven drops (0·2 ml.) of suitably diluted bacterial suspensions were delivered from calibrated dropping pipettes on to the surface of agar plates, and spread by rotation. Plates were incubated at 37°, either in a "humid" incubator or in sealed jars containing a pad of moistened cotton-wool.

Dilutions were made in 0·9 per cent NaCl or 1 per cent phosphate buffer pH 7·6; broth is not superior to these simple diluents.

Haematin estimations were made by the pyridine-haemochromogen method of Keilin and Hartree (1935) and Rimington (1942).

Strains.—Five strains of *Past. pestis* were available. Three of them (No. 327, 336 and 337) were highly virulent (Herbert, 1947); they had been preserved by drying soon after primary isolation from human plague cases in N. Africa. Strains "Soemedang" and "Tjiwidej" were the completely avirulent "live vaccine" strains of Otten (1936). Stocks were preserved in the dried state by the gelatin-ascorbic acid technique of Stamp (1947); cultures in current use were grown in peptic blood agar stabs and kept at 0°, subcultures being made monthly for not more than six months.

RESULTS.

Necessity of a substance present in peptonized blood for aerobic growth of isolated cells.

The basic phenomenon under investigation is illustrated in Table I and Fig. 1. Two series of Evans' peptone-agar plates, to one of which pepsinized sheep's blood had been added, were seeded with serial dilutions of an 18-hour broth culture of *Past. pestis*, and incubated aerobically at 37°. With high dilutions (less than 650 cells per plate) no colonies developed on the plain agar plates in 40 hours, and less than 6 per cent of the expected number developed even after 5 days' incubation; on the plates with added peptic blood, however, all the colonies were fully developed in 40 hours (and were just visible in 24 hours). With very heavy inocula producing confluent growth there was no visible difference between the two series. The peptic blood, therefore, is only necessary for the development of widely isolated single cells.

Table II shows that under *anaerobic* conditions peptic blood is not required, and the full number of colonies develops on plain agar. Under semi-anaerobic conditions, as in poured plates incubated in air, a proportion of the cells develops in plain agar, but not the full number. Plates containing 25 ml. of agar were used, and it could be seen that colonies only developed deep in the agar. Under

TABLE I.—*Effect of Pepsinized Sheep's Blood on Growth of Small Inocula on Agar Plates.*

Dilution of broth culture; 1 in—	Evans peptone. Colonies visible in—		Evans peptone plus 5% pepsinized sheep's blood. Colonies visible in—	
	40 hours.	5 days.	40 hours.	5 days.
10	Confluent	Confluent	Confluent	Confluent
4×10^5	0	33	*ca.* 600	Semi-confluent
8×10^5	0	19	324	324
16×10^5	0	7	148	148
32×10^5	0	3	70	70
64×10^5	0	3	35	35

Evans peptone agar plates, with or without pepsinized sheep's blood, seeded with 7 standard drops (0·2 ml.) of the indicated dilution of an 18-hour broth culture of strain "Tjiwidej," and incubated aerobically at 37°.

TABLE II.—*Effect of Pepsinized Blood in Surface and Poured Plates, under Aerobic and Anaerobic Conditions.*

		Colonies visible in 40 hours.	
		Evans peptone.	Evans peptone + 5% pepsinized blood.
Aerobic	Surface plates	0	375
	Poured plates	34	343
Anaerobic	Surface plates	362	354
	Poured plates	352	369

All plates seeded with 7 standard drops (0·2 ml.) of a suspension of strain "Tjiwidej" and incubated at 37°, either in air or in a McIntosh and Fildes anaerobic jar.

anaerobic conditions the colonies are smaller, more flattened, and take longer to develop than those produced aerobically in the presence of peptic blood (Fig. 1), but the same number of colonies develops in both cases.

In ordinary media, therefore, *Past. pestis* behaves as if it were an anaerobe, while in the presence of pepsinized blood it grows aerobically or anaerobically.

Identification of the active substance in pepsinized blood.

The nature of the substance in peptic blood responsible for the above effects was now investigated. Fildes (1920, 1921) who first used peptic blood for the cultivation of *Haemophilus influenzae*, showed that its growth-promoting effect was due to two substances, (1) haematin, and (2) a heat-labile factor, later named "V-factor" and eventually identified as triphosphopyridine-nucleotide. It was found that autoclaving the peptic blood did not affect its growth-promoting action for *Past. pestis*, which immediately suggested that the active substance might be haematin. Small amounts of haemin were then added to the basal agar medium and were found to promote the aerobic growth of isolated cells in exactly the same way as peptic blood (Tables III to V). Commercial samples of haemin and a highly purified specimen had quantitatively identical effects.

These experiments alone do not prove that the effect of the peptic blood is entirely attributable to the haematin it contains. To test this point, the haematin

content of a sample of peptic blood was determined by the pyridine-haemochromogen method, and a series of plates containing falling concentrations of peptic blood was compared with a second series containing equivalent amounts of pure haemin. The results (Table III and Fig. 1) show that the effect of peptic blood can be entirely accounted for by its haematin content. In both series 100 per cent of colonies developed with 5 μg. haemin/ml., and *ca.* 15 per cent developed with 1 μg./ml. ; with sub-optimal amounts of haemin or peptic blood the colonies were irregular in size as well as deficient in numbers (Fig. 1).

With large amounts of peptic blood (>2 per cent) colonies are visible earlier, and are definitely larger after 40 hours than with equivalent amounts of pure haemin, though the same number of colonies develops. This is probably due to the presence in peptic blood of other substances having a generalized accelerating effect on growth ; with lower concentrations of peptic blood the effect is hardly noticeable.

TABLE III.—*Comparison of Pepsinized Sheep's Blood and Equivalent Amounts of Haemin.*

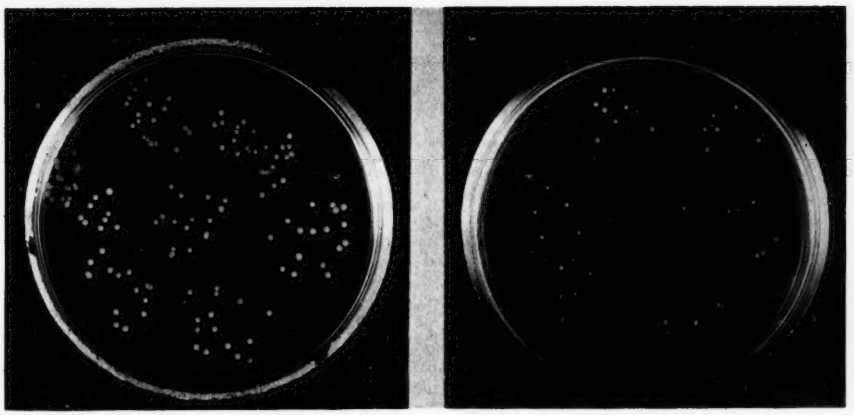
Additions to basal medium.	Colonies developing in 40 hours.
Haemin 10 μg./ml	141
,, 5 ,,	149
,, 2 ,,	55
,, 1 ,,	29
,, 0·5 ,,	2
1% pepsinized sheep blood (10·5 μg. haemin/ml.)	146
0·5% ,, ,, ,, (5·25μg. ,,)	134
0·25% ,, ,, ,, (2·01 μg. ,,)	107
0·1% ,, ,, ,, (1·05 μg. ,,)	17
0·05% ,, ,, ,, (0·525 μg. ,,)	1
No additions	0
,, grown anaerobically	138

Basal medium tryptic meat agar, containing the indicated addition of pure haemin, or pepsinized sheep's blood of known haemin content. All plates seeded with 7 drops (0·2 ml.) of the same suspension of strain " Tjiwidej " and incubated at 37° in air, or in anaerobic jars.

TABLE IV.—*Effect of KCN and Haemin under Aerobic and Anaerobic Conditions.*

	Number of colonies developing in 40 hours.			
	Aerobic.		Anaerobic.	
KCN conc. (M).	No haemin.	Haemin. 10 μg./ml.	No haemin.	Haemin. 10 μg./ml.
0	0	370	340	350
0·0002	0	186	348	356
0·001	0	36	402	351
0·004	0	0	385	395
0·02	0	0	325	327

The basal medium was tryptic meat agar, containi ıg the indicated additions of haemin or KCN (neutralized). All plates seeded with 7 standard drops (0·2 ml.) of a suspension of strain " Tjiwidej," and counted after 40 hours' incubation at 37°.



Haemin 5 μg./ml.
Aerobic

Haemin 1 μg./ml.
Aerobic

No Additions
Anaerobic

No Additions
Aerobic

FIG. 1.—Effect of haemin, and anaerobiosis, on growth of *Pasteurella pestis* on agar plates. Basal medium tryptic meat agar, with indicated additions of haemin. All plates received the same inoculum (*ca.* 140 cells) of a 24-hour culture of strain "Tjiwidej," and were incubated 40 hours at 37° C.

TABLE V.—*Effect of Various Substances on Aerobic Growth from Small Inocula.*

Additions to basal medium.	Colonies developing in 40 hours.		
	Exp. 1.	Exp. 2.	Exp. 3.
Nil	0	0	0
5% pepsinized sheep's blood	242	259	195
Haemin (10 μg./ml.)	238	—	—
Thiolacetic acid (0·1%)	220	—	—
Na_2SO_3 (0·1%)	—	278	—
Charcoal (B.D.H. "blood charcoal")	—	238	—
Maize starch (0·2%)	0	—	—
Glucose (0·5%)	—	—	0

Basal medium tryptic meat agar. Thiolacetic acid and Na_2SO_3 sterilized by filtration, remaining substances by autoclaving. All plates in each experiment seeded with 7 drops of the same suspension, and incubated aerobically at 37°.

The "haematin effect" is independent of the basal medium used, identical results being obtained with tryptic meat digest agar, Evans' peptone, and casein hydrolysate.

All of the five strains tested (3 highly virulent and 2 completely avirulent) showed the "haematin effect."

Absence of haematin in ordinary bacteriological media.

The above experiments show the effect of haematin to be maximal at 5 μg./ml. and detectable at 1 μg./ml.; since no growth at all occurred on the basal media they presumably contain less than 1 μg. haematin/ml. In the case of tryptic digest agar this is unexpected, since the meat from which it is made is rich in haematin compounds. Most of the haematin is removed during the preparation of the medium, with the coagulum formed when the digested meat is steamed at pH 6; even so, it seems surprising that small traces do not remain.

The probable explanation was discovered when a batch of medium was encountered on which isolated colonies grew without added haemin. It was found that in its preparation the usual "dephosphating" procedure, warming at pH 8·6 to precipitate calcium phosphate, and filtration, had accidentally been omitted. Another batch of medium was prepared, half of which was dephosphated while the remainder was not. Agar plates made from the dephosphated broth gave no aerobic growth of *Past. pestis* unless haemin was added. Plates made from the broth that had not been dephosphated gave submaximal growth without addition of haemin, about 50 per cent of the expected number of colonies developing; this corresponds to the effect produced by adding about 2 μg. haemin/ml. to haemin-free media.

A model experiment was carried out by adding haemin (10 μg./ml.) to tap-water (containing *ca.* 100 mg. calcium/litre) adjusted to pH 8·6, then adding M/100 phosphate of the same pH, and centrifuging. Quantitative estimations showed that all the haematin had been carried down on the calcium phosphate precipitate, no detectable amounts remaining in the supernatant.

Pappenheimer and Shaskan (1944) removed traces of iron from simplified media by adding calcium salts and phosphate, all the inorganic iron being adsorbed on the calcium phosphate precipitate. Evidently the same technique

will be useful when it is desired to render complex media completely free from haematin.

Effect of other substances on aerobic growth from small inocula.

These results suggest that (*a*) *Past. pestis* can synthesize haematin with difficulty or not at all, and (*b*) that haematin might be the prosthetic group of some enzyme which is necessary for aerobic growth. The effect of KCN, which inhibits iron-porphyrin enzymes such as catalase, peroxidase and cytochrome oxidase, was therefore tested (Table IV). Anaerobically, KCN in concentrations up to M/250 had no effect, and M/50 a scarcely significant effect, whether haemin is present or absent. Aerobically, no colonies developed unless haemin was present, but if KCN was also present it abolished the effect of haematin completely at M/250, by 90 per cent at M/1000 and by 50 per cent at M/5000. (The actual concentrations present after 40 hours were probably less than these, owing to the volatility of HCN.).

These results agree with the above hypothesis. The position became more complicated, however, when other substances were tested in place of haemin. It was then found (Table V) that sodium sulphite, thiolacetic acid and animal charcoal would promote the aerobic growth of isolated colonies in exactly the same way as peptic blood and haemin ; starch and glucose, however, had no effect. Charcoal and starch were tested because of their stimulating action on the growth of gonococci and meningococci (Glass and Kennett, 1939 ; Mueller and Hinton, 1941).

It should be pointed out that haemin is effective in concentrations at least 100 times lower than the other active substances tested.

Experiments on chemically defined media.

The above results are open to various interpretations, and it was thought that a clearer insight into the factors concerned would be gained by studying the nutrition of *Past. pestis*, and conducting experiments in a chemically defined medium. Three workers (Rao, 1939 ; Berkman, 1942 ; Doudoroff, 1943) have already studied this problem, but their results are so conflicting that it was necessary to approach the matter afresh.

It was found that none of the five strains available would grow on the simple media containing only two or three amino-acids described by Rao (1939) or Doudoroff (1943). The next medium tested was similar to that of Berkman (1942), containing 20 amino-acids, ammonia, salts and glucose. With this medium, results were irregular. Usually no growth occurred, but occasionally some flasks grew up after 3–5 days' incubation. This effect was erratic, e.g. in duplicate flasks sometimes one would grow when the other did not, and usually growth only occurred when a heavy inoculum was used, e.g. 10^6 cells/ml. It was concluded either that only a very small proportion of cells in any culture was capable of growth in this medium, or else that actual mutation or " training " was sometimes occurring.

Table VI shows the effect of adding haemin, and mixtures of 17 other growth factors, to the amino-acid medium. No growth occurred with the amino-acids alone, or with the 17 other growth factors, while good growth occurred with haemin. 5μg./ml. of haemin gave a maximum effect and 1μg./ml. a detectable

TABLE VI.—*Growth in Amino-acid Medium; Effect of Haemin and Other Growth Factors.*

Additions to amino-acid medium.	Growth after incubation at 37° for—			
	24 hours.	40 hours.	48 hours.	3 days.
Nil	0	0	0	0
Haemin 1 μg./ml.	0	Trace	±	++
,, 2 ,,	0	+	+±	++
,, 5 ,,	0	++	+++	++±
,, 10 ,,	0	++	+++	++±
Growth factors I	0	0	0	0
,, ,, I + II	0	0	0	±
,, ,, I + haemin (10 μg./ml.)	0	+++	+++	++±
,, ,, I + II + haemin (10 μg./ml.)	Trace	+++	+++	++±

Ten ml. complete amino-acid medium with M/100 glucose, salts and the indicated additions, inoculated with 1250 viable cells/ml. Strain 337. Growth Factors Group I: Nicotinamide, 5×10^{-6} M; pyridoxin, pantothenic acid, riboflavine, 2×10^{-6} M; p-aminobenzoic acid, thiamine, 10^{-6} M; biotin 10^{-3} g./ml. Group II: Glutamine, 2×10^{-4} M; choline, uracil, adenine, guanine, thymine, xanthine, inositol, 5×10^{-5} M; oleic acid, 2×10^{-5} M; pimelic acid, 5×10^{-6} M.

effect—exactly the same concentrations as those found effective by the agar plate technique (Table III). When the 17 growth factors were added in addition to haemin, growth was slightly, but consistently, faster than with haemin alone. Further experiments showed that no single growth factor alone had any noticeable effect, while various mixtures of them had slight accelerating effects which appeared to be additive.

The conclusion was that *Past. pestis* is capable of synthesizing all these growth factors, but will grow rather faster if they are supplied in the medium. The effect of haemin was of quite a different order, since without it growth was always greatly delayed, and usually did not occur at all, particularly if small inocula were used. The amino-acid medium with haemin consistently supported growth with inocula as small as 10 cells/ml. Strain 337 was still highly virulent for mice after 13 sub-cultures in this medium.

All of the five strains tested grew on this medium. The amino-acid requirements of one of them, virulent Strain 337, were studied in some detail. The effect was first tested of omitting each of the amino-acids singly from the complete amino-acids-haemin medium. The results showed that the amino-acids fall into four groups:

(1) *Omission from the medium has no effect*—glycine, alanine, *nor*leucine, aspartic and glutamic acids, lysine, proline, hydroxyprolone, tyrosine and tryptophane.

(2) *Omission causes slight delay in growth*—serine, methionine, arginine.

(3) *Omission causes marked delay in growth*—threonine, histidine.

(4) *Omission causes complete absence of growth*—valine, leucine, isoleucine, cystine and phenylalanine.

From this experiment, which was repeated with identical results, most of the amino-acids appeared to be dispensable and only those of Group 4 to be "essential." However, a mixture of the five "essential" amino-acids with ammonia, glucose and haemin did not support growth, even when threonine, histidine and

TABLE VII.—*Growth on Mixtures of Amino-acids.*

Amino-acid mixture.	Growth after incubation at 37° for— 16 hours.	24 hours.	40 hours.	48 hours.
(1) Valine, leucine, isoleucine, cystine, phenylalanine	0	0	0	0
(2) As 1 + threonine and histidine	0	0	0	0
(3) As 2 + proline	0	0	0	0
(4) As 3 + glycine, alanine and glutamic acid	0	Trace	+	++
(5) As 4 + serine and methionine	0	±	++	+++
(6) Complete amino-acid medium	Trace	++	++±	+++

Amino-acids in same concentrations as previous experiments. All mixtures contained M/100 glucose, salts and haemin (5 μg./ml.). Inoculum 10^5 cells/ml. Strain 337.

proline were also added (Table VII). Further addition of glycine, alanine and glutamic acid gave moderate growth, and with these plus serine and methionine the growth was fairly good, though still slower than on the complete amino-acid medium. This may not be the simplest possible mixture adequate for growth, but it is evident that the minimum number of amino-acids required is not small.

The other four strains were not investigated in detail. All required some amino-acids, since they would not grow on an ammonia-salts-glucose medium, even when haematin and 17 other growth factors were added. None required tryptophane, since growth occurred on an acid-hydrolysed casein medium (with glucose and haemin) known by microbiological tests to be tryptophane-free.

These results show that for growth under the conditions employed, which are fairly aerobic, thin layers of liquid in Erlenmeyer flasks, *Past. pestis* requires a number of amino-acids and haemin. Table VIII shows the effects of haemin under aerobic and strictly anaerobic conditions. It will be seen that anaerobically, growth is equally rapid whether haemin is presen tor absent, while aerobically no growth occurs without haemin. Haemin can be replaced, however, by the reducing agent thiolacetic acid, in whose presence aerobic growth occurs without haemin as it does anaerobically.

Comparison of these results with those of Tables III to V shows that the effects of haemin, reducing agents and anaerobiosis are essentially the same in a chemically defined liquid medium as they are on nutrient agar plates.

TABLE VIII.—*Effect of Haemin, Thiolacetic acid, and Anaerobiosis, on Growth in Amino-acid Medium.*

Additions to amino-acid medium.	Aerobic. 24 hours.	48 hours.	3 days.	Anaerobic. 24 hours.	48 hours.	3 days.
Nil	0	0	0	0	++±	++±
Thiolacetic acid (M/100)	0	++±	+++	0	++±	++±
Haemin (5μg./ml.)	Trace	+++	+++	0	++±	++±

Ten ml. complete amino-acid medium with M/100 glucose, salts, and indicated additions inoculated with 2000 cells/ml. "Aerobic" in air/5 per cent CO_2; "Anaerobic" in hydrogen/5 per cent CO_2 in McIntosh and Fildes anaerobic jar. Strain 337.

Catalase.

Bacterial catalase, like that of higher animals, is a haematin-protein (Herbert and Pinsent, 1948). Bacteria unable to synthesize haematin are therefore unable to synthesize catalase unless haematin is supplied in the growth medium.

All strains of *Past. pestis* tested produced an active catalase when grown in the amino-acids-glucose medium under the following conditions :

(*a*) Aerobic or anaerobic growth in the presence of haemin.

(*b*) Anaerobic growth in the absence of haemin.

(*c*) Aerobic growth without haemin in the presence of reducing agents, such as thiolacetic acid.

(*d*) On the rare occasions when growth was observed, usually from large inocula, in the absence of either haemin or a reducing agent.

In short, whenever the organisms grew at all, they produced catalase, whether haemin was supplied in the growth medium or not. The same was true of growth on agar media.

Ability to synthesize catalase in the absence of added haematin shows that the organisms can themselves synthesize haematin. The bearing of this on the " haematin effect " is discussed below.

DISCUSSION.

The nutrition of *Past. pestis* has been studied by three previous workers, with rather discrepant results. Berkman (1942) found that four out of five old laboratory cultures grew in a medium containing 15 amino-acids and glucose, without added growth factors. Rao (1939) claimed that a medium consisting only of the three substances cystine, phenylalanine and proline was sufficient to support growth. Doudoroff (1943) found that five strains would grow in an ammonia-salts-glucose medium with cystine and phenyl-alanine added. None of these authors found haemin to be essential for growth, though Rao reported that it had an accelerating effect.

None of the strains tested in this work would grow in media as simple as those of Rao or Doudoroff. Anaerobically they would all grow in a mixture of 20 amino-acids and glucose, but for aerobic growth haemin was also required, at least when small inocula were used. This medium supported growth from inocula as small as 10 cells per ml. For one virulent strain whose requirements were investigated in detail, at least five amino acids were needed.

Some of these discrepancies may be due to strain differences, but they are probably mainly due to differences in size of inocula. Rao used very heavy inocula, 0·2 ml. per 10 ml. tube of a suspension of opacity 1 on Brown's Scale, and stated that inocula of 7000 viable cells per tube would not grow. Doudoroff stated, " Initially large inocula had to be used, and visible turbidity was slow to appear, evidence pointing to a selection of the cells most capable of reproducing in this environment." The larger the inoculum, the greater the chance of including rare " mutants " with minimal growth requirement, and in liquid medium the development of only one cell out of the whole inoculum will be recorded as positive growth. Dr. G. M. Hills informs me that he has found some strains of *Past. pestis* to be nutritionally more exacting when grown at 37° than at 28° ; this may partly account for the varied findings of different workers as far as

amino-acid requirements are concerned. However, I have observed the "haematin effect" on solid media to occur equally at 37° and at 30°.

It seems probable that this is the reason why, in my experiments, growth was occasionally observed from large inocula in liquid synthetic media without haemin, while previous workers, using even larger inocula, have missed the "haematin effect" altogether.

From this point of view the use of solid media is more satisfactory, and allows the "haematin effect" to be particularly clearly demonstrated, since the actual number of organisms in the inoculum which develop may be counted. The ideal technique, no doubt, would be the use of chemically defined solid media; however, results so far obtained show fairly clearly that the effect of haematin is essentially the same in liquid synthetic media and complex solid media.

A complete explanation of the "haematin effect" is not yet possible. One hypothesis considered was that hydrogen peroxide, produced by the bacteria under aerobic but not anaerobic conditions, was inhibiting aerobic growth. The possibility of hydrogen peroxide originating from the growth medium might apply to tryptic digest agar, but presumably not to amino-acid media. The effect of haematin might then be ascribed to its utilization by the organism for the synthesis of catalase; since HCN is a strong inhibitor of catalase, this could account for its reversal of the effect of haematin. Animal charcoal, which can be substituted for haematin, catalytically decomposes hydrogen peroxide. The effect of reducing substances such as sulphite or thiolacetate could be ascribed either to direct chemical reaction with hydrogen peroxide, or to their reaction with oxygen in the medium producing effectively anaerobic conditions, so that hydrogen peroxide is not formed.

This explanation seems to be controverted by the fact that *Past. pestis* can synthesize catalase in the absence of added haematin. This objection may be only apparent. Measurements of the catalase content of bacteria at different stages of the growth cycle (Pinsent, 1949) show that it is low in the early stages of growth and often does not reach its maximum until after growth has ceased. In the development of isolated single bacteria it is probably the first few cell-divisions that are crucial, and it may well be that at this stage the cell has insufficient catalase for its requirements under aerobic conditions.

A simpler, if vaguer, hypothesis is that growth depends on the oxidation-reduction potential of the medium, and can only take place if the E_h is below a certain level. This could explain most of the observed phenomena, but it is difficult in this way to account for the effect of added haematin, for it seems unlikely that, in a complex medium, haematin concentrations of a few μg./ml. could directly affect the E_h.

However, until more facts are known, it will remain easier to devise alternative hypotheses than to confirm or disprove them.

SUMMARY.

Heavy inocula of *Pasteurella pestis* grow aerobically on the surface of ordinary agar media, but widely separated single cells will not grow. Isolated cells will grow, however, if whole blood or a peptic digest of blood are added to the agar.

The factor in peptonized blood responsible for this effect was identified as haematin; the effect can be duplicated by the addition to plain agar of pure

recrystallized haemin, which has a maximum effect at a concentration of 5 μg./ml. and a detectable effect at 1 μg./ml. The effect of haemin is abolished by the simultaneous addition of M/1000 KCN.

This action of haemin is not a simple growth-factor effect, for under anaerobic conditions isolated cells will grow on plain agar without haemin, and growth is not inhibited by KCN. Also, isolated cells will grow aerobically in the absence of haemin if blood charcoal or reducing substances such as sodium sulphite or thiolacetate are added to the agar, though much higher concentrations of these are needed than of haemin.

The nutritional requirements of *Past. pestis* have been studied in chemically defined media. This organism requires none of the ordinary growth-factors, and grows anaerobically in a medium containing only synthetic amino-acids, salts and glucose. A preliminary study of the amino-acid requirements has been made.

For aerobic growth from small inocula in amino-acid media, haemin or a reducing agent such as thiolacetate must be added, confirming the results obtained with complex agar media.

As a preliminary hypothesis it is suggested that hydrogen peroxide is produced under aerobic conditions, and inhibits growth. Aerobic growth is made possible by adding reducing substances, which may act either by direct chemical reaction with hydrogen peroxide, or by rendering growth-conditions effectively anaerobic so that no peroxide is produced. Added haemin is assumed to act by promoting the synthesis of bacterial catalase.

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SEROLOGICAL STUDIES WITH HERPES SIMPLEX VIRUS.

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THE detection and measurement of antibody to herpes simplex virus in the serum of experimental animals or man has generally been made by neutralization tests. In these tests mixtures of serum and active virus have been injected into rabbits by the intracerebral route (Zinsser and Tang, 1929) or intradermally (Andrewes and Carmichael, 1930) or intracerebrally into mice (Weyer 1932, Hudson, Cook and Adair, 1936). In 1939 Burnet and Lush carried out neutralization tests on the chorio-allantois of developing chick embryos. These methods give satisfactory results, but are expensive in both time and material. Bedson and Bland (1929) showed that herpetic antibody could be detected in the serum of immunized guinea-pigs by the complement-fixation test, using extracts of herpes-infected guinea-pig pads as antigen ; by this technique antibody in human sera was demonstrated by Brain (1932), who found that approximately 45 per cent of sera from unselected hospital patients gave a positive reaction. Other workers have apparently not been able to obtain consistently satisfactory results by this *in vitro* method (Myers and Chapman, 1937), and in two recent American works on virus diseases only the neutralization test is recommended for the detection of antibody to herpes simplex virus in human sera (Scott, 1948 ; Scott, Coriell and Blank, 1949).

In the course of the present work the results of Bedson and Bland (1929) were readily confirmed in that antibody to herpes simplex virus in the serum of immunized animals could readily be measured by their complement-fixation technique. As we had available several strains of herpes virus which had been adapted to the chorio-allantois, the tissues and fluids from infected chick embryos have been examined for their suitability as antigens in the complement-fixation test. As shown by Anderson (1940), egg adapted strains of herpes virus produce generalized infection of chick embryos ; after inoculation of such strains into the yolk sac or amniotic cavity, virus is present in high concentration in the amniotic fluid several days later (Nagler, 1946 ; Beveridge and Burnet, 1946). Extracts of herpes-infected chorio-allantoic membranes, amniotic and allantoic fluids have been successfully used in the present work as antigens in complement-fixation tests.

MATERIAL AND METHODS.

Virus strains.

Four strains of herpes virus have been studied. Strain " N " was isolated directly in chick embryos by the inoculation of the chorio-allantois with vesicle fluid from a case of a generalized infection with herpes simplex virus (Kipping and Downie, 1948). The other three strains, " H," " McC " and " P," have been

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isolated within the last 12 months from members of the staff who suffer from recurrent attacks of labial herpes. In each case the virus was isolated without difficulty by the inoculation of the chorio-allantois of developing eggs with diluted vesicle fluid.

Preparation of the antigens.

In the inoculation of chick embryos by various routes the techniques described by Beveridge and Burnet (1946) have been followed.

Chorio-allantoic membrane extracts.

Twelve-day-old embryos were inoculated on the chorio-allantois and the membranes were removed after two days' further incubation. They were ground up in a tube with powdered glass by means of a glass rod and then extracted with saline containing 20 per cent nutrient broth, approximately 0·5 ml. being added for each membrane. After being kept, with occasional shaking, for 2 hours at room temperature, the suspension was centrifuged at low speed until the supernatant fluid was clear (5–10 min.). The supernatant was removed and used as the antigen.

Extra-embryonic fluids.

Allantoic and amniotic fluids were harvested within 24 hours after the death of the embryo following either yolk sac, amniotic or allantoic inoculation with a suspension in broth of herpes-infected chorio-allantoic membrane.

Antisera.

Immune human serum against three strains, " H," " McC " and " P," were obtained from the individuals who furnished the virus strains. Immune sera were prepared against each strain by immunization of guinea-pigs. To avoid the formation of antibody to chick protein herpes-infected guinea-pig pads were used as the source of inoculum in each case. The infected pads were ground finely and approximately 5 ml. of sterile nutrient broth were added. The suspensions were treated with sufficient penicillin, and in the case of strain " H " with streptomycin as well, so that subsequent cultures on agar and in broth showed no bacterial growth. Each preparation contained sufficient virus to produce a confluent growth of typical herpetic lesions on the chorio-allantois. The strain " N " had undergone 30 previous egg-to-egg passages on the chorio-allantois, strains " H," " McC " and " P " had undergone 15, 7 and 4 respectively. Guinea-pigs " H," " P " and " N " each received their first two inoculations intradermally into the plantar skin of both hind pads at an interval of two days. On the 4th day after the primary inoculation the soles of the hind feet were swollen and vesicles were developing along the needle tracks. On the 8th day " N " had developed paralysis of both hind limbs, from which it never completely recovered. The animals each received a subcutaneous inoculation of virus on the 31st day, and two further intraperitoneal inoculations on the 52nd and 59th days after the primary inoculation. A week later guinea-pig " N " appeared extremely excitable and was bled out from the heart. Animals " H " and " P " each received two further intraperitoneal injections on the 66th and 73rd days, and were bled a week later. Guinea-pig " McC " received its first inoculation

intradermally into the plantar skin of both hind pads at the same time as the other animals. As the feet showed signs of an early "take" after two days, it was not given a further inoculation at this time. A week later there was paralysis of the hind limbs, but this had cleared up within a fortnight. On the 37th day after the primary inoculation the animal was given a subcutaneous injection of virus. Fourteen days later the right hind leg was badly inflamed and the animal was bled. Immune rabbit serum had been prepared against strain "N" by immunization with active virus obtained from infected rabbit testicle (Kipping and Downie, 1948). Immediately before use all sera were inactivated by heating at 56° C. for half an hour.

Technique of Complement fixation Tests.

The unit volume of reagents used was 0·2 ml. Antigen dilution, complement and serum were mixed and kept in the refrigerator at 4° overnight (18 hours). Next morning the tubes were kept at room temperature for 30 minutes before adding 0·4 ml. of sensitized sheep red cell suspension (equal volumes of 2 per cent red cell suspension and amboceptor diluted to contain 3 M.H.D.). The tubes were kept in a water bath at 37° for 30 minutes and then at room temperature for 1–2 hours to allow red cells to settle before the readings were made.

When extracts of chorio-allantoic membrane were used as antigen 2½ M.H.D. of complement was used in the tests, but with amniotic or allantoic fluid antigens 2 M.H.D. was used. All serum and antigen control tubes received ½ dose less complement than the tests proper. Tests with extra-embryonic fluids or chorio-allantoic membrane extracts from normal uninoculated eggs were set up with all experiments. Serum from normal guinea-pigs or rabbits was similarly tested with immune sera from these species, and negative human serum from one of several non-herpetic subjects on the staff of the department was used to control the tests with human sera.

RESULTS.

Complement fixation with Antigens Prepared from Infected Chorio-Allantoic Membranes, Allantoic and Amniotic Fluids.

The first table shows the results with chorio-allantoic antigen, falling dilutions of the antigen being tested with falling dilutions of immune human serum.

TABLE I.—*Titrations of Chorio-Allantoic Membrane Extract with Dilutions of Immune Human Serum.*

Antigen dilutions.	Dilutions of immune serum.				Negative human serum.		No serum.
	1/2.	1/4.	1/8.	1/16.	1/2.	1/4.	
1/4	++++	++++	++++	+++	−	−	−
1/8	++±	+++	+++±	+++	−	−	−
1/16	++	++	++±	++	−	−	−
1/32	+	+	+	+	−	−	−
1/64	−	−	−	−	−	−	−

++++ = Complete fixation. − = No fixation.

+++, ++, + Indicate degrees of fixation less than complete.

Similar results were obtained using immune rabbit serum except that the rabbit serum had a higher antibody content than the human serum, as shown by the higher dilution of serum which gave positive reactions.

Table II shows the results when human serum diluted 1/8 was used to test falling dilutions of allantoic and amniotic fluids.

TABLE II.—*Titration of Allantoic and Amniotic Fluids with Immune Human Serum diluted* 1/8.

Antigens	Immune human serum 1/8. Dilutions of antigens. 1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	Negative human serum 1/8. 1/4.	1/8.	No serum. 1/4.
Amniotic fluid	++++	++++	++++	++++	+++	−	−	−	−
Allantoic fluid	++++	++++	++++	++±	−	−	−	−	−
Normal fluid	−	−	−	−	−	−	−	−	−

Table III shows the results when falling dilutions of immune guinea-pig sera "McC" and "N" were titrated against constant antigen—allantoic fluid diluted 1/8.

TABLE III.—*Titration of Immune Guinea-pig Sera with Constant Antigen (Allantoic Fluid diluted* 1/8).

Sera.	Infected allantoic fluid 1/8. Dilutions of guinea-pig sera. 1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	Norman allantoic fluid. 1/8.	1/16.	No antigen. 1/8.
"McC"	++++	++++	++++	++±	−	−	−	−	−
"N"	++++	++++	++++	++++	++	−	−	−	−
Normal	−	−	−	−	−	−	−	−	−

The constant dilution of antiserum or antigen used for titration of antigen and antiserum respectively as shown in Tables II and III were chosen, on the bases of preliminary tests of the kind shown in Table I, as those likely to give high titres.

The Nature of the Antigen and its Stability to Heat and Storage.

The activity of the three antigens—allantoic fluid, amniotic fluid and chorio-allantoic membrane extract—appears to be related not to the virus particle itself but to some "soluble" specific substance separable from the virus by centrifugation.

To elicit this point the antigens were spun in an angle centrifuge at approximately 8000 r.p.m. for one hour. The upper half of the supernatant was carefully removed, the lower half was discarded, care being taken not to disturb the deposit. The tube was well drained, and the deposit resuspended in saline containing 20 per cent nutrient broth. The centrifugation was repeated, the supernatant fluid removed as before, and the deposit again resuspended to its original volume.

The complement-fixing power of the original fluid, 1st supernatant, 2nd supernatant and resuspended deposit were tested against immune human or guinea-pig sera.

Tests of the infectivity of the original fluid and 1st supernatant, determined by titration on the chorio-allantoic membranes of developing chick embryos, showed that the 1st supernatant contained less than 2 per cent of the virus present in the original fluid.

Table IV shows the results of testing various fractions of allantoic fluid with immune human serum diluted 1/8.

TABLE IV.—*Titration of Various Fractions of Infected Allantoic Fluid with Immune Human Serum* (1/8).

Antigens	Immune human serum (1/8). Dilutions of antigens.							Negative human serum (1/8).				No serum.	
	Undiluted.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	Undiluted.	1/2.	1/4.	1/8.	Undiluted.	1/4.
Original fluid	0	0	++++	++++	++++	++++	+	0	0	—	—	0	—
First supernatant	0	0	++++	++++	++++	++++	—	0	0	—	—	0	—
Second supernatant	—	—	—	—	—	0	0	—	—	0	0	—	0
Resuspended deposit	—	—	—	—	—	0	0	—	—	0	0	—	0

0 = Not tested.

An essentially similar result was obtained when infected amniotic fluid and mixtures of amniotic and allantoic fluids were tested in the same way against a constant dilution of immune human serum.

Examination of an antigen prepared from infected chorio-allantoic membrane gave the results shown in Table V. The infectivity of this antigen was not tested. However, titration of another membrane extract similarly prepared showed that approximately 98 per cent of the virus was deposited during the first centrifugation. It will be seen that the resuspended washed deposit showed more fixation of complement than similar deposits from amniotic and allantoic fluid antigens. This difference has been apparent in several experiments of this kind, and suggests that soluble antigen is more difficult to remove from the tissue particles deposited with the virus from extracts of infected chorio-allantois.

TABLE V.—*Titration of Various Fractions of Chorio-Allantoic Membrane Extract with Immune Human Serum* (1/8).

Antigens.	Immune human serum 1/8.					Negative human serum 1/8.			No serum.	
	Dilutions of antigen.									
	1/2.	1/4.	1/8.	1/16.	1/32.	1/2.	1/4.	1/8.	1/2.	1/4.
Original fluid	0	++++	++++	+++	++	0	−	−	0	−
First supernatant	++++	++++	+++	++	−	−	−	0	−	0
Second supernatant	−	−	−	−	−	−	−	0	−	0
Resuspended deposit	+++	++	−	−	−	−	−	0	−	0

0 = Not tested.

The effects of heating the " soluble " antigen at 56° for one hour and of boiling for 5 minutes are shown in Table VI. The antigen used was the first supernatant from the high speed centrifugation of infected allantoic fluid. It is apparent that the complement-fixing activity is greatly weakened by heating at 56° for 1 hour, but is not completely destroyed even by boiling for 5 minutes.

TABLE VI.—*Effect of Heat on the " Soluble " Antigen.*

Antigens.	Immune human serum 1/8.						Negative human serum 1/8.		No serum.	
	Dilutions of antigen.									
	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/2.	1/8.	1/2.	1/8.
Unheated	0	0	++++	++++	++++	−	0	−	0	−
Heated 56° 1 hour	++++	++++	+++	−	−	−	−	0	−	0
Boiled 5 min.	++++	±	−	−	−	−	−	0	−	0

0 = Not tested.

An infected allantoic fluid was tested after storage for three months in a refrigerator at approximately 4°. Using the same immune human serum for test before and after storage there appeared to have been little, if any, fall in the

complement-fixing titre of the fluid during this time. The effect of treatment of the antigen with formalin has not been investigated, but it appears that infected chick embryo fluids, when stored at low temperature, may be used for the titration of antibody in sera over a period of several months.

A Serological Comparison of Four Recently Isolated Strains of Herpes Virus by Complement-Fixation Technique.

There is very little evidence in the literature of immunological diversity among strains of herpes virus. Florman and Trader (1947) studied four strains of diverse origin; using immune sera prepared in rabbits they found that, by neutralization tests in mice and on the chorio-allantois, three strains appeared to be immunologically identical, while the fourth was only partially neutralized by the sera prepared against the other three strains. Slavin and Gavett (1946) similarly found that one of three strains differed from the other two in neutralization tests in mice using immune rabbit sera.

Sera from the individuals who provided three of the herpes strains available to us were used in tests of amniotic fluid antigens from all four strains. The fluids were used in a dilution of 1/4 in two instances and 1/8 in the other two; the results are shown in Table VII.

The three sera when tested against allantoic fluid antigens gave essentially the same results as those shown in Table VII. The results with the sera from immunized guinea-pigs are shown in Table VIII.

Although two of the guinea-pig sera were very weak in complement-fixing antibody it is clear that these tests, like the tests with the human sera, give no indication of strain specificity among the four viruses.

It is perhaps of interest that the two weak guinea-pig antisera were obtained

TABLE VII.—*Titration of Immune Human Sera "H," "McC" and "P"*

Sera.	Antigens (diluted "H" 1/4.	"H" 1/8.	"H" 1/16.	"H" 1/32.	"McC." 1/4.	"McC." 1/8.	"McC." 1/16.	"McC." 1/32.
"H"	++++	++++	++++	++	++++	++++	++++	+
"McC"	++++	++++	++	−	+++±	+++±	±	−
"P"	++++	++++	++	+	++++	++++	++	−
Negative serum	−	−	0	0	−	−	0	0

TABLE VIII.—*Titration of Immune Guinea-pig Sera "H," "McC*

Sera.	Antigens (dilu "H" (allantoic fluid 1/8). 1/4.	1/8.	1/16.	1/32.	1/64.	1/80.	"McC" (amniotic fluid 1/8). 1/4.	1/8.	1/16.	1/32.	1/64.	1/80.
"H"	+	+	++±	−	−	−	−	+	+±	+	−	−
"P"	±	−	−	−	−	−	±	−	−	−	−	−
"McC"	0	0	++++	++++	++++	−	0	0	++++	++++	++++	−
"N"	0	0	++++	++++	++++	+	0	0	++++	++++	++++	++++±
Negative	−	−	0	0	0	0	−	−	0	0	0	0

from animals that had received a full course of inoculations with living virus comprising two intradermal, one subcutaneous and four intraperitoneal injections over a period of approximately ten weeks. Neither of those two animals showed evidence of paralysis. The two animals which gave good complement fixing antisera, on the other hand, had a smaller number of immunizing injections, but both showed paralysis of the hind limbs following the pad inoculations.

The Complement fixation Test for the Detection of Antibody in Human Sera.

Various workers have shown that neutralizing antibody for herpes virus is present in a large proportion of adult sera, and that in general those showing such antibody have a history of recurrent herpetic infection. Only Brain (1932) appears to have used the complement-fixation technique in such work. He found that of 52 unselected sera from London hospital patients 23 gave complement fixation with herpetic antigens. We have tested 40 sera which had been sent to the Liverpool City Laboratory for Wassermann test. Of these sera only seven were negative in the complement-fixation test with a mixed allantoic and amniotic herpetic antigen. These sera were tested in a dilution of 1/4 and greater. Of the 33 which gave a positive reaction all but one (which was positive 1/32) were positive in dilutions up to 1/64, the titres being 1/64 or 1/128. The results bore no relation to the results of the W.R. The sera could hardly be regarded as representative specimens from the general population, but the percentage of positives (82·5), although higher than that recorded by Brain, is not higher than the figure given by Burnet and Lush (1939) for neutralizing antibody in the sera of hospital patients in Melbourne. The results in this series also show the same all or nothing kind of result in that sera were either completely negative or had high complement fixing titres.

against Antigens (Amniotic Fluids) "H," "P," "McC" and "N."

1/4 or 1/8).

"P"				"N."				Normal amniotic fluid.		No antigen.
Serum dilutions										
1/4.	1/8.	1/16.	1/32.	1/4.	1/8.	1/16.	1/32.	1/4.	1/8.	1/4.
++++	++++	++++	++	++++	++++	++++	++	−	−	−
++++	++++	±	−	++++	++++	±	−	−	−	−
++++	++++	+	−	++++	++++	+	−	−	−	−
−	−	0	0	−	−	0	0	−	−	−

P" and "N" against Antigens "H," "McC," "P" and "N."

or 1/8).

"P" (allantoic fluid 1/4).					"N" (amniotic fluid 1/8).						Normal allantoic fluid.		Normal amniotic fluid.		No. antigen.
rum dilutions															
R.	1/15.	1/32.	1/64.	1/80.	1/4.	1/8.	1/16.	1/32.	1/64.	1/80	1/4.	1/8.	1/4.	1/8.	1/4.
+	+	±	−	−	−	+	+	±	−	−	−	−	−	−	−
−	−	−	−	−	±	−	−	−	−	−	−	−	−	−	−
0	++++	++++	++++	±	0	0	++++	++++	++±	−	−	−	−	−	−
0	++++	++++	++++	++±	0	0	++++	++++	++++	++	−	−	−	−	−
−	0	0	0	0	−	−	0	0	0	0	−	−	−	−	−

DISCUSSION.

These experiments have shown that satisfactory herpetic antigens for complement-fixation tests with immune sera from man or animals can be obtained from the amniotic or allantoic fluids of chick embryos infected with the virus of herpes simplex by various routes. The strains of virus used yielded satisfactory antigens after a few passages on the chorio-allantois of chick embryos, by which time it was found that generalized infection of the embryos regularly following inoculation by the yolk sac or amniotic routes. The antigen in allantoic and amniotic fluids is on the whole more satisfactory than that prepared from infected chorio-allantoic membranes, as the fluids are almost devoid of anti-complementary properties. The complement-fixing antigen may be obtained relatively free from virus and is relatively heat-labile, being greatly weakened by heating at 56° for one hour. Fluids, after boiling, may retain slight complement-fixing properties with immune sera, suggesting that there may be a more stable component of the antigen; in the experiments described, however, such heat-stable antigen was apparently of little importance in the reactions obtained with unheated fluid.

The experiments recorded afford no proof that the complement-fixing antibody in immune sera is the same as that concerned with neutralization of the virus. However, ten sera were tested both by the complement-fixation technique and by neutralization of herpes virus on the chorio-allantois; there was complete correlation of the results obtained by the two methods. Of 13 individuals whose history as regards herpetic infection was known, the sera from all those, and only from those, with such a history gave a positive complement-fixation test. Further, the proportion of positives found by complement-fixation on sera sent for routine Wassermann tests was similar to that recorded by neutralization tests in the hands of other workers, and the results on individual sera were similarly either completely negative or strongly positive. No weak positive or doubtful reactions were encountered.

SUMMARY.

Complement-fixing antigen has been demonstrated in suspensions of chorio-allantoic membrane 2 days after inoculation with herpes simplex virus and in extra-embryonic fluids within 24 hours after the death of the embryo following either yolk sac, allantoic or amniotic inoculation. Sera from immunized guinea-pigs and rabbits and sera from persons liable to recurrent herpetic infection served for the demonstration of the antigen.

The activity of the complement-fixing antigen is chiefly due to a "soluble" specific substance separable from the virus by high-speed centrifugation.

The activity of the "soluble" antigen is largely destroyed by heat at 56° C. for 1 hour; a slight degree of activity is still retained after boiling for 5 minutes. The antigen can be stored with little loss at 4° for 3 months.

Using the complement-fixation technique, no antigenic differences could be demonstrated among 4 recently isolated strains of herpes virus.

The complement-fixation technique affords a sensitive method for detecting herpetic antibody in human sera, and is more economical of time and materials than neutralization tests.

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CIRCULATING FREE AMINO-ACIDS IN HYPOGLYCAEMIA

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IN a previous communication (Mayer-Gross and Walker, 1949) it was shown that the intravenous injection of 20 g. of L glutamic, aminoacetic or para-aminobenzoic acid to subjects in hypoglycaemic coma could result in the restoration of consciousness, and that, while some rise in the blood glucose occurred in such cases, it was insufficient to account for the return of consciousness.

In search of an explanation of this effect of the parenteral introduction of excessive amino-acids into the blood, it seemed of interest to consider the influence of hypoglycaemia on the normally circulating free amino-acids. It had long been known that the free amino-acids in the blood are diminished in hypoglycaemia (Luck, Morrison and Wilbur, 1928; Daniels and Luck, 1931; Harris, Blalock and Horwitz, 1938; Harris and Harris, 1947), and that the subsequent administration of glucose results in a further decrease in the circulating amino-acids (Luck, Davis and Van Winkle, 1935; Harris *et al.*, 1938). Davis and Van Winkle (1934) and Crisman, Hanvey and Luck (1940) have ascribed this to the effect of adrenalin secreted in response to the injected insulin, because they found that the decrease in blood amino-acid does not occur when insulin is injected into the adrenalectomized animal, and that the injection of adrenalin itself to the intact animal produced hypoaminoacidemia. If adrenalin is the agent which causes the diminution of amino-acids one would expect to find a definite relation between the amino-acid and blood glucose levels indicating the sympathomimetic action of adrenalin. Moreover, the amino-acid content in subjects exhibiting greater restlessness and less depth of coma should differ from that in subjects who progressively go into a quiet and deep coma.

There are, however, other possible explanations of the amino-acid decrease in hypoglycaemia besides adrenalinaemia. Daniels and Luck (1931) have discussed the hypothesis that insulin hypoaminoacidaemia may be due to an increased rate of glycogenesis from amino-acids, the hypoglycaemic state providing a stimulus to glycogenesis. They also thought of a more direct influence of insulin on protein metabolism. That this exists has been confirmed by more recent work. Bouckaert and his collaborators (Laquet, de Nayer and Bouckaert, 1934; Bouckaert and de Duve, 1947) and Mirsky (1938) have shown that insulin exerts an inhibitory effect on proteolysis in the peripheral tissues as well as that it promotes or inhibits deamination in the liver, the latter being dependent on the blood glucose level.

Some of these observations have a bearing on our special problem, and proved accessible to study on clinical material. In the first part of the present paper serial experiments are reported demonstrating a quantitative relationship between plasma amino-acid and blood glucose levels in hypoglycaemia. In the second place, the effect of adrenalinaemia on the amino-acid level is examined, using

clinical observations as a test without special regard to the blood glucose level. Finally, an attempt is described to test hepatic deamination using galactose as an indicator of the speed of glycogenesis in the presence and absence of excessive amino-acid in the circulation.

TECHNICAL.

The subjects of the experiments were, as previously, patients undergoing Sakel's Insulin Coma Treatment and the same standard of coma was adopted. An injectable preparation of L glutamic acid was made as described before (Mayer-Gross and Walker, 1949), and blood glucose determinations were made by the method of King, Haselwood and Delory (1937). Plasma amino-acid nitrogen determinations were made by the method of Folin as modified by Danielson, Sahyun and Frame, Russell and Wilhelmi (Hawk, Oser and Summerson, 1947). Blood galactose was estimated by the same technique as blood glucose, with the exception that the isotonic sodium sulphate solution contained a fine suspension of washed yeast, and that the blood was allowed to remain in contact with this for ten minutes before precipitation of the proteins took place. Under these conditions the blood glucose was completely fermented while the galactose was not affected. By the use of standard solutions of galactose it was possible to compile a table of relationship between N/200 sodium thiosulphate solution and galactose.

EXPERIMENTS AND RESULTS.

Plasma Amino-acid and Blood Glucose.

It was desirable to obtain comprehensive data of the concentrations of plasma amino-acid nitrogen, blood glucose and of the subject's state of consciousness over the whole period of "Insulin Treatment," from the point at which insulin was first given to that at which consciousness was fully restored after either a sucrose feed alone or combined with an intravenous injection of glucose.

A series of 22 experiments was carried out in which simultaneous estimations of plasma amino-acid nitrogen and blood glucose were made before the injection of insulin and at hourly intervals thereafter until the point of onset of coma. Observations were made at this point and 30 minutes later, immediately before the administration of 200 g. of sucrose by a stomach-tube, furthermore at the point at which consciousness was restored and after a further 20 minutes. If consciousness was not restored within 30 minutes of the administration of the sucrose the blood glucose and the plasma amino-acid nitrogen were estimated before the intravenous injection of 33 per cent (W/V) glucose solution. The quantity injected varied from one case to another, but averaged 80 ml. (25 g.). After a further period of 15 minutes the observations on blood and plasma were repeated.

The results of these experiments are set out in Table I, in which Columns 6 and 7 are alternative to Columns 8 and 9, depending upon whether or not it was necessary to give an intravenous injection of glucose in order to restore consciousness. In 19 of the 22 cases it will be observed that up to the point of the onset of coma the concentration of plasma amino-acid nitrogen moves in the same direction as that of the blood glucose and the degrees of consciousness, and that it was less at the end of coma than at the start in 12 of the 22 cases. Columns

TABLE I.—*Concentration in mg./100 ml. of Plasma Amino-acid Nitrogen (AAN_2) and Blood Glucose (Gl) before the Injection of Insulin and at Hourly Intervals Thereafter Until the Point of Onset of Coma; after 30 Minutes of Coma and (Col. 6 and 7) at the Moment of Restoration of Consciousness after a Tube Feed of 200 g. of Sucrose and 20 Minutes Later or (Col. 7 and 8), if Consciousness had not been Restored, 30 Minutes after the Feed of Sucrose and 15 Minutes after an Intravenous Injection of Glucose.*

Patient.	1. Before insulin.		2. 1 hour.		3. 2 hours.		4. Coma onset.		5. 30 min. coma.		6. Awake.		7. 20 min. later.		8. 60 min coma.		9. 15 min. after i.v. glucose.	
	AAN_2.	Gl.	AAN_2.	Gl.	AAN_2.	Gl.	AAN_2.	Gl.	AAN_2.	Gl.	AAN_2.	Gl.	AAN_2.	Gl.	AAN_2.	Gl.	AAN_2.	Gl.
Tw.	7·5	70	7·3	44	5·7	17	5·7	7	5·7	7	4·8	34	6·0	83	—	—	—	—
Wy.	7·4	70	7·0	58	4·5	20	5·7	12	4·6	9	—	—	—	—	4·2	34	4·4	197
Cr.	7·2	81	6·1	25	6·0	14	4·6	12	3·8	12	—	—	—	—	4·0	30	4·8	86
Th.	6·9	63	6·3	24	5·7	14	5·9	7	5·0	7	4·8	48	5·6	81	—	—	—	—
Ru.	9·0	58	8·2	14	—	—	7·4	7	6·9	5	—	—	—	—	5·7	46	6·5	125
Fa.	6·7	72	6·2	52	6·0	24	5·0	17	5·0	12	5·4	39	5·1	65	—	—	—	—
Fl.	8·6	81	7·8	23	—	—	7·6	12	5·5	7	—	—	—	—	5·4	35	6·3	128
Jo.	9·2	56	8·2	27	7·4	21	7·0	14	6·0	12	—	—	—	—	4·1	9	6·0	163
Th.	7·5	54	6·1	21	—	—	5·4	11	6·3	7	—	—	—	—	4·7	17	5·7	98
Ne.	6·5	62	5·5	18	—	—	5·2	11	5·9	11	5·4	40	5·0	75	—	—	—	—
Fl.	6·6	72	4·8	25	—	—	3·8	17	4·1	12	4·0	47	3·7	70	—	—	—	—
Cr.	5·2	95	5·0	25	4·2	23	4·2	14	4·5	9	—	—	—	—	4·4	28	4·8	200
Co.	7·2	77	5·5	23	4·8	9	4·5	9	4·4	9	4·0	23	3·3	65	—	—	—	—
Tw.	7·1	55	6·6	28	6·0	23	4·0	12	4·2	12	—	—	—	—	3·7	28	5·6	127
Fa.	7·0	75	6·1	52	6·0	23	5·7	12	3·3	7	3·2	46	5·9	104	—	—	—	—
Ni.	6·8	86	6·4	21	4·1	18	4·5	9	4·5	7	3·8	51	4·5	90	—	—	—	—
Ne.	9·0	69	8·2	29	—	—	6·2	13	7·3	7	5·4	52	6·3	87	—	—	—	—
Th.	7·3	63	7·1	23	—	—	5·5	11	5·3	6	—	—	—	—	5·1	23	6·4	104
Mn.	7·2	72	4·7	21	4·9	14	3·9	14	4·1	12	3·7	42	3·4	51	—	—	—	—
Cr.	5·5	86	5·4	37	5·2	23	4·1	14	4·1	14	4·0	51	3·7	58	—	—	—	—
Fl.	7·2	77	5·7	14	5·3	9	5·2	7	5·2	14	4·9	44	4·5	67	—	—	—	—
Cr.	7·8	102	6·3	21	5·4	21	5·3	12	5·5	15	4·7	40	4·3	46	—	—	—	—

6, 7, 8 and 9 show that the administration of carbohydrate either in the form of sucrose by mouth alone or together with an intravenous injection of glucose frequently resulted in a further decrease in the plasma amino-acid nitrogen. Examination of the figures in these columns, however, shows that although some of the amino-acid figures fall, others rise. When the corresponding blood glucose level is less than approximately 80 mg./100 ml. the plasma amino-acid continues to fall, and over that level the amino-acid concentration commences to rise.

If such a critical level of blood glucose exists below which hypoaminoacidemia takes place, this had to be demonstrated in an experimental series with more critical observations, in which the blood glucose level during coma was varied by giving small amounts of intravenous glucose. In eight patients the first couple of blood specimens were taken two hours after the injection of sufficient insulin to produce a state of hypoglycaemic coma, and thereafter at four 15-minute intervals after the intravenous injection of 5 g. of glucose, and at two 15-minute intervals after the subsequent injection of 30 g. glucose. The results are set out in Table II. In one case, Wy., Column 3, an increase in the plasma amino-acid nitrogen was noted at a blood glucose level of 12 mg./100 ml., and in five observations it remained constant despite a considerable degree of hypoglycaemia. All the remaining observations, 42 in number, were in agreement with the principle noticed in the previous experiment. That the blood glucose levels in Column 7 are in some cases higher than those in Column 6 is explained by the fact that after the injection of 30 g. of glucose a quantity of sucrose dilution was given as a drink for the patient's benefit.

The figures from Columns 6, 7, 8 and 9 of Table I and from Columns 2, 3, 4, 5, 6 and 7 of Table II are combined in the scatter diagram, Fig. 1. which demonstrates the manner in which the rise and fall of the plasma amino-acid nitrogen is related to the level of the blood glucose, and in particular to the level of 80 mg./100 ml.

Plasma Amino-acid and Behaviour in Coma.

There are marked individual variations of the subject's behaviour in hypoglycaemic coma. Some reach the stage quietly and remain so while the unconsciousness deepens progressively during the 30 minutes before interruption. In others the depth of coma fluctuates first and only later progressive deepening takes place. Others again show much restlessness, myoclonic twitchings and

TABLE II.—*Concentration in mg./100 ml. of Plasma Amino-acid Nitrogen (AAN_2) and of Blood Glucose (Gl) in Hypoglycaemic Patients Before and After the Successive Intravenous Injection of 5 and 30 g. of Glucose.*

Patient.	1. 2 hours after insulin.		2. 15 min. after 5 g. glucose.		3. 30 min. after 5 g.		4. 45 min. after 5 g.		5. 60 min. after 5. g.		6. 15 min. after 30 g.		7. 30 min. after 30 g.	
	AAN_2.	Gl.	AAN_2.	Gl.	AAN_2	Gl.	AAN_2.	Gl.	AAN_2.	Gl.	AAN_2.	Gl.	AAN_2.	Gl.
Tc.	5·4	19	4·6	48	4·6	25	4·5	21	4·0	23	5·3	120	7·5	130
Col.	4·4	23	5·5	92	4·4	25	4·4	23	4·3	16	4·9	87	5·1	130
Tw.	5·1	28	5·0	35	5·0	28	4·9	28	3·6	23	4·9	90	6·9	107
Wy.	5·9	12	4·8	14	6·2	12	5·6	12	5·6	9	6·2	93	7·1	95
McN.	5·5	12	4·1	23	3·8	19	3·2	12	2·9	12	5·7	132	6·0	130
Fl.	4·4	14	3·7	21	3·6	21	3·4	14	3·5	12	4·3	111	4·9	97
Th.	5·3	9	3·9	14	3·8	9	3·6	7	3·0	9	5·4	118	6·1	130
Co.	4·2	21	4·0	48	3·9	35	3·8	25	3·7	14	4·9	109	5·4	104

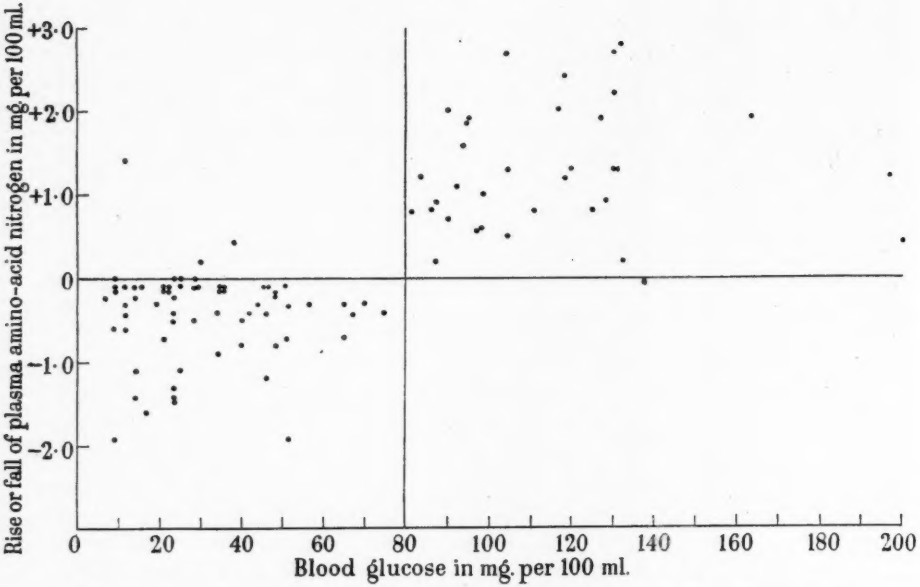

FIG. 1.—Showing the rise or fall in plasma amino-acid nitrogen after the injection of glucose to hypoglycaemic subjects plotted against the resultant blood glucose concentration.

extrapyramidal movements. The fluctuations in depth are generally believed to be caused by the sympathomimetic effect of adrenalin counteracting the insulin. Restlessness increases the output of adrenalin into the circulation.

Both phenomena can be enhanced by sensory stimulation—for instance by needle-pricks in the withdrawal of blood. If adrenaemia is, as was suggested, responsible for the lowering of the amino acid level, different values should be found parallel to the variations in the subject's behaviour in coma.

In a series of fifteen experiments estimations of the plasma amino-acid nitrogen were made approximately 10 minutes before the patient became comatose, at the time at which coma commenced and at intervals of 10, 20 and 30 minutes thereafter. Notes regarding the patients' state of consciousness and behaviour were made at the same times. The results are summarized in Table III and show that in 13 of the 15 cases there is an average fall of 0·9 mg./100 ml. in plasma amino-acid nitrogen at the time of the onset of coma. This fall is not always maintained, and is in several cases followed by a rise. In several cases where the coma was quiet, i.e. not complicated by variations in depth or by restlessness, there is a progressive fall, but in other similar cases this is not so. On the other hand, the first case, although complicated by great fluctuations in the depth of coma, shows a progressive fall in the concentration of plasma amino-acid nitrogen. Three cases where excessive restlessness characterized the course of the coma, Numbers 2, 4 and 8, show a general tendency for the plasma amino-acid nitrogen to rise.

TABLE III.—*Concentration of Plasma Amino-acid Nitrogen in mg./100 ml. 10 Minutes Before the Onset of Hypoglycaemic Coma, at the Moment of the Onset of Coma and at Intervals of 10, 20 and 30 Minutes Thereafter.*

Number.	10 min. before coma.	Coma onset.	10 min. coma.	20 min. coma.	30 min. coma.
1	5·5	4·8	4·4F	4·2F	3·9
2	4·6	3·0R	3·3R	3·6	3·9
3	6·1	4·6R	3·9	5·1	4·7
4	3·5	4·6R	5·0R	4·8	5·4
5	4·5	3·8F	4·8F	5·0F	4·1
6	5·7	5·0	5·7F	5·0	4·4
7	5·5	4·7	4·8F	5·6F	6·1
8	5·0R	5·4R	5·5RF	5·5RF	5·7
9	5·7	5·5	4·6R	5·0	5·4
10	4·8	3·8	3·9	5·2	4·8
11	4·7	4·6	4·4	4·2	4·2
12	4·7	4·6	4·6	4·4	4·3
13	4·5	3·6	3·6	4·5	5·0
14	5·9	3·6	5·6	5·1	4·5
15	5·6	4·4F	4·5	4·0	5·2

F = Fluctuating coma; R = considerable restlessness.

This series of experiments failed to show any direct relationship between the plasma amino-acid nitrogen and the behaviour of the subjects in hypoglycaemic coma. The theory that the decrease in circulating amino-acids in hypoglycaemia is due to the action of adrenalin was not confirmed, since in those restless cases in which the output of adrenalin might be expected to be high the fall in the plasma amino-acid nitrogen was often the least marked.

Iufluence of Excessive Amino-acids on Glycogenesis.

Since the most probable reason for the withdrawal of free amino-acids from the circulation in hypoglycaemia is their deamination in the liver, and in view of the fact that this appeared to be reversed by the elevation of the blood glucose above 80 mg./100 ml., it appeared to be worth while to examine a complementary phenomenon, i.e. the effect of an abnormal amount of circulating amino-acid upon the rate of formation of glycogen in the presence of an excess of insulin. For this purpose the rate of disappearance of galactose was observed when injected alone and in the presence of an excess of L-glutamic acid. Galactose is converted into glycogen relatively slowly, and cannot be directly utilized in the tissues (Peters and Van Slyke, 1946).

Approximately two hours after the injection of sufficient insulin to produce hypoglycaemic coma, nine patients received intravenously 20 g. of L-glutamic acid as well as 25 g. of galactose. After these injections parallel observations were made of blood glucose, blood galactose and plasma amino-acid nitrogen at intervals of 15, 30, 45, 60 and 90 minutes. On a subsequent day a control experiment was carried out on the same subjects, receiving the same dose of insulin, who had been given only 25 g. of galactose. Blood glucose and galactose were estimated at the same intervals.

The results are set out in Table IV, those of the experiments with glutamic acid in Columns 1–7 and those of the controls with galactose alone in Columns 8–14. When L-glutamic acid was injected the galactose concentration was greater and its persistence in the blood longer than in the control experiment. As an expression of concentration and persistence the "Galactose Index" is given for the experiments and controls in Columns 7 and 14 respectively. The figure was arrived at from the formula :

$$\frac{\text{Sum of galactose concentrations in mg./100 ml.} \times \text{time of persistence in minutes}}{100.}$$

Its ratio between the experimental and control values is never less than 2 : 1, and in 6 of the 9 cases is greater than 5 : 1.

Columns 1–6 of Table IV also show a reasonably constant relationship between the blood level of amino-acid nitrogen and the presence of circulating galactose. With the exception of the second case, CZ., galactose is not absent from the circulation while the concentration of plasma amino-acid nitrogen is substantially above that existing before the injection of L-glutamic acid and galactose. In Cases 1, 6, 7, 8 and 9 circulating galactose persists throughout the period of the experiment, and in each case the amino-acid nitrogen remained above the level originally observed. Similarly the glucose values were higher in the presence of injected glutamic acid than in its absence.

If the glucose values from Columns 2–6 (experiments) and 9–13 (controls) are added up in each patient, one finds that in 6 of the cases the ratio is approximately 2 : 1 and in one case nearly 5 : 1 ; in the remaining two cases the figures are approximately equal. Thus, in 7 of the 9 cases the quantity of circulating glucose under the experimental conditions was double that under those of the controls.

DISCUSSION.

If hypoglycaemia induced by an excess of insulin is regarded as a state of emergency, during which the homeostatically so very important level of blood glucose is artificially lowered, one finds that the different organs of the human body respond in a set order to this emergency. In earlier work we were able to show that, for instance, the cerebral cortex only becomes unresponsive, producing the state defined as coma, when the blood glucose was decreased to a level of 10 to 15 mg./100 ml. (Mayer-Gross and Walker, 1945). Depression of the function of the taste buds controlling the taste for sweetness already takes place at a blood glucose level of 50 mg./100 ml. (Mayer-Gross and Walker, 1946). Below this level our subjects selected from a number of drinks a concentrated sucrose solution, which they rejected because of its excessive sweetness above this level.

The result of the first series of our present experiments suggests another critical blood glucose level : while plasma amino-acid decreases progressively under the influence of insulin as long as the blood glucose remains below 80 mg./100 ml., this process is stopped and reversed when it rises above this level. Because of the further fall of amino-acid after the oral intake of sucrose and after waking up from coma, glucose and amino-acid levels seemed to be unconnected ; in fact the mechanisms are linked up closely.

If one tries to explain this finding, one has to consider the three relations

TABLE IV.—*Concentration in mg./100 ml. of Plasma Amino-acid Nitrogen (AAN_2), Blood Galactose (Gal) and Blood Glucose (Gl) Before and After the Intravenous Injection of 25 g. of Galactose and 20 g. Glutamic Acid to Hypoglycaemic Patients (Experiments) and of Blood Glucose and Galactose After the Intravenous Injection of 25 g. of Galactose Alone to the Same Subjects (Controls).*

Experiments.

Patient.	1. Before galactose and glutamic acid.			2. 15 min.			3. 30 min.			4. 45 min.			5. 60 min.			6. 90 min.			7. Galactose index.
	AAN_2.	Gal.	Gl.	AAN_2.	Gal.	Gl.	AAN_2.	Gal.	Gl.	AAN_2.	Gal.	Gl.	AAN_2.	Gal.	Gl.	AAN_2.	Gal.	Gl.	
Bm.	7·0	—	21	16·6	78	67	14·1	66	49	11·5	44	44	9·6	24	35	7·2	6	25	196
Cz.	4·9	—	14	11·7	30	30	10·1	12	25	10·1	0	23	7·9	0	16	4·8	0	12	13
Wh.	5·7	—	14	12·1	37	19	9·9	30	14	9·7	26	12	8·9	26	7	6·6	0	12	71
Og.	6·0	—	35	11·0	26	51	9·7	22	25	8·4	7	32	7·4	4	23	5·6	0	25	35
Be.	6·3	—	19	15·2	67	32	14·5	30	30	10·9	19	25	8·3	19	19	6·0	0	23	81
Co.	4·5	—	16	15·0	74	14	11·0	41	21	9·4	19	23	6·6	19	19	5·7	7	7	144
Mn.	4·5	—	21	11·3	74	21	10·1	52	25	8·5	37	12	6·6	33	9	5·3	7	21	183
Cl.	3·9	—	12	12·0	115	19	11·1	74	16	10·2	52	19	8·7	37	19	7·6	30	12	277
Hu.	5·5	—	12	14·1	74	28	13·1	52	23	11·9	33	21	9·3	26	16	7·7	19	16	248

Controls.

Patient.	8. Before galactose.		9. 15 min.		10. 30 min.		11. 45 min.		12. 60 min.		13. 90 min.		14. Galactose index.
	Gal.	Gl.	Gal.	Gl.	Gal.	Gl.	Gal.	Gl.	Gal.	Gl.	Gal.	Gl.	
Bm.	—	9	32	12	7	12	0	9	0	7	0	7	12
Cz.	—	9	19	19	5	17	0	12	0	7	0	7	7
Wh.	—	16	11	25	0	16	0	9	0	9	0	12	2
Og.	—	30	19	35	7	33	0	31	0	33	0	21	8
Be.	—	19	33	12	15	9	7	9	0	9	0	12	25
Co.	—	9	26	7	11	7	0	12	0	9	0	9	11
Mn.	—	12	60	12	24	9	0	12	0	9	0	9	25
Cl.	—	9	52	25	41	9	19	7	0	9	0	9	50
Hu.	—	14	52	21	37	9	19	7	0	9	0	9	49

between insulin and circulating amino-acids mentioned in our introductory remarks. Of these adrenalaemia can, from our observations, be dismissed as an explanation. The second series of our experiments undertaken to test the effect of adrenalin fluctuations gave no confirmation of an interdependence of adrenalinaemia, as observed clinically, and amino-acid level in the blood. Moreover, the minor blood glucose variations well known as indicators of adrenalinaemia are not mirrored in the amino-acid values—an observation adding further weight to the clinical evidence.

The inhibitory effect of insulin on proteolysis in the peripheral tissues may contribute to the lowering of the circulating amino-acids ; but it is difficult to see how this should be linked up with the critical blood glucose level as closely as we found it. If an excess of insulin is responsible for the inhibitory effect, such an excess is still present in our patients for some time after they have recovered from hypoglycaemia, as shown by their tendency to relapse into mild hypoglycaemia during the afternoon of the same day. Nevertheless, hypoaminoacidaemia does not persist after the blood glucose is above the critical level.

The most plausible explanation is, therefore, that hepatic deamination for the purpose of glyconeogenesis is the main cause of the observed decrease of amino-acids—a process which one would expect to be controlled by the blood glucose level. The experiments of Laquet *et al.* (1934), who found that in animals receiving small amounts of glycine insulin raised the blood amino-acid level if the blood glucose level was maintained by infusion of glucose, are strongly in support of such a control. So is the work of Ashby (1949) who has shown that the intraperitoneal administration of amino-acids to the adrenalectomised rat diminishes the rate of conversion of glucose to liver glycogen.

To gain further insight into the interrelation between amino-acid circulating in the blood and the formation of glycogen was the object of our third experimental series. The great difference between the values for the " galactose index " under the two sets of conditions shows that the rate of glycogen formation must be markedly affected by the presence of the excess amino-acid. The fact that the galactose persists in the circulation while the amino-acid concentration remains above normal is confirmatory evidence.

The result of this as well as that of the earlier experiments can also throw some light on our primary problem, viz. the waking effect of excessive amino-acids in hypoglycaemic coma. It has become clear why no direct relationship exists between the spontaneous hypoaminoacidaemia and the state of consciousness in hypoglycaemia ; for at the blood glucose level of 80 mg./100 ml., at which hypoaminoacidaemia terminates, consciousness has been restored for a long time. The brain, as explained before, returns to normal function at a much lower blood glucose level. Hence also the inefficacy of fluctuations of consciousness on the circulating amino-acids in a condition below the critical level (Table III).

The galactose experiments, on the other hand, suggest that the acute raising of the amino-acid level in the blood not only stimulates hepatic deamination of amino-acids, but at the same time slows down glycogenesis from circulating carbohydrates. Some of the circulating glucose which otherwise would be the main source of glycogenesis is thus saved. It may be that this glucose, although not raised in its actual level, persists in the circulation for a longer time and becomes more readily available to the brain cell—a hypothesis which will be tested in further experiments.

SUMMARY.

When plasma amino-acid nitrogen was observed before and after the injection of insulin in subjects undergoing Sakel's hypoglycaemia treatment, it was found to decrease as long as the blood glucose content was below 80 mg./100 ml. If glucose was administered, this decrease was reversed only if the blood glucose rose above this critical level.

The theory that adrenalin is responsible for the fluctuations in circulating amino-acids was not confirmed by clinical observations. Serial experiments failed to show any relationship between behaviour of the subjects in hypoglycaemia and amino-acid level in the plasma.

Hepatic deamination being one of the possible causes of hypoaminoacidaemia, galactose was used as an indicator of the interdependence of the level of circulating amino-acids and of glycogenesis from circulating carbohydrate. Observations of its concentration and persistence in the blood of hypoglycaemic subjects was compared in the presence and absence of an excess of L-glutamic acid injected at the same time. The results strongly suggested the existence of such an interdependence.

The experiments are discussed considering previous findings of other critical blood glucose levels in hypoglycaemia, and in view of the explanation they may provide for the waking effect of excessive amino-acids in hypoglycaemic coma.

We wish to express our gratitude to Dr. P. K. McCowan, Physician Superintendent of Crichton Royal, for his interest in our work and for his permission to publish the results. Our thanks are also due to Miss A. Beattie, sister-in-charge, and to the other members of the staff of the Insulin Department, for their collaboration.

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THE FAILURE OF ANTI-HISTAMINE DRUGS TO INFLUENCE THE LOCAL VASCULAR CHANGES IN EXPERIMENTAL BURNS.

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WHILST there is general agreement that the erythema of burns is due to the dilatation of the skin capillaries and venules, and that the oedema and blister fluids of more severe burns arise from the blood plasma via the walls of these tiny vessels, the mechanism of these phenomena is still in doubt. Ebbecke (1923) postulated that a chemical substance was liberated from damaged tissues and acted on the local capillaries. Lewis (1927) developed this theory and asserted that this compound was histamine or a histamine-like substance, the H-substance. In more severe burns the oedema, blistering and peripheral extension of the erythema beyond the burn site, the " flare," were regarded by Lewis as being due to the release of larger amounts of H-substance which acted both directly on the vessels and by stimulation of local axon reflexes. This theory could not be critically tested until drugs with a powerful anti-histamine action were discovered and experiments designed to test the truth of this hypothesis by the use of such drugs are recorded in this paper.

PERMEABILITY AND BLOOD FLOW CHANGES IN GUINEA-PIG BURNS.

The methods used in detecting these changes have been described elsewhere (Sevitt, 1949) and are summarized below.

If the circulating plasma of an animal is dyed by the injection of Evans Blue (T1824) or Brilliant Vital Red (Colour Index 456) and a burn is inflicted, the development of a dyed patch of skin indicates the flow of blood through skin vessels of increased permeability. This has been called the *patch* reaction (Fig. 1) and occurs in the less severe burns whether the dye is introduced before burning or some hours later. In more severe burns *stasis* of the dermal blood flow occurs and can be demonstrated by introducing the dye some time after burning. A narrow blue ring appears at the periphery of the burn, but the burn area itself remains erythematous. In this *ring-with-erythema* reaction, the persistant erythema which fails to blanche on pressure, indicates the presence of stasis, and the peripheral coloured ring indicates graded vascular damage at the edge of the burn.

In some ring-with-erythema reactions, dye slowly moves inwards from the coloured peripheral ring, encroaching upon and obscuring the central erythema. This is called the *temporary ring-with-erythema* reaction and indicates extreme retardation or *stagnation* of the blood flow.

Double dye technique.

Continuation of the blood flow over a period, or the development of stasis within a period, may be demonstrated by the introduction of the red and blue dyes at different times. For examples two 20-seconds burns, at 60° and 65° C. respectively, were inflicted on a guinea-pig. An hour later Brilliant Vital Red was injected intracardially and red patch reactions developed in both burns (Upper and lower burns in Fig. 1). Three hours later, Evans Blue was introduced. In the 60° C. burn, the red patch was replaced by a blue one and this indicated the continuation of the dermal blood flow and the continued permeability change over the four-hour period (Upper burns in Fig. 3): in the 65° C. burn, however, a narrow ring appeared at the periphery of the red circular patch which otherwise remained unchanged. This indicated that stasis had occurred later than 1 hour after burning and within the following 5 hours (Lower right burn in Fig. 3).

PRESENT INVESTIGATION.

To determine whether anti-histamine drugs could modify their course, several burns varying in severity but of known temperature and duration were inflicted on each of a group of guinea-pigs. Half of these were previously injected with one or other of the drugs β-dimethylaminoethyl benzhydryl ether hydrochloride (Parke Davis & Co.), 2-phenylbenzyl aminomethyl-imidazoline (Ciba Ltd.) or N-dimethylaminoethyl-N-p-methoxybenzyl-a-aminopyridine acid maleate (neo-antergan) (May & Baker, Ltd.). These will be referred to as benadryl, antistin and anthisan respectively. The permeability and blood flow changes of each burn were determined by the dye technique and the results were compared, burn for burn, in the two sets of animals. Other comparisons made were the degree of oedema, the presence of erythema under the site of application of the burning iron and the development of cyanosis therein, the retention or loss of sensation in the burned skin and the subsequent depth of skin loss. Four sets of experiments were performed and in none of them were important differences noted between the burns on the control and the anti-histaminized animals.

EXPERIMENTAL.

Albino guinea-pigs (560 to 670 g.) were burned under open-ether anaesthesia by firmly pressing the burning surface of the iron on to the depilated back or abdomen. The iron (Sevitt, 1949) was essentially a hollow brass cylinder through which circulated a current of hot water, the temperature of which could be varied and recorded. The cylinder was closed at one end, and this, the burning surface, was circular and measured 1 in. in diameter.

Evans Blue and Brilliant Vital Red, both 1 per cent solutions, were injected intracardially in a dosage of about 1 c.c.

Sensibility was determined at intervals by pinching the burned skin with a forceps and noting the reaction of the animal.

By examining the burns every 3 to 5 days, each was graded into one of the categories of no skin loss, superficial skin loss, partial skin loss or whole skin loss.

CONTROL OF THE EXPERIMENTS.

Preliminary experiments with, and dosage of, anti-histamine drugs.

When 0·05 c.c. of 0·05 per cent histamine acid phosphate was injected into the depilated skin of a normal guinea-pig which had previously been injected intracardially with Evans Blue, a well marked blue wheal 9 to 12 mm. in diameter occurred. This procedure was adopted as the routine skin histamine test.

Half to one hour after the intramuscular injection of 50 mg. of antistin (70 to 90 mg. per kg.) into a guinea-pig the skin histamine test failed to produce a wheal and the inhibitory effect generally lasted 3 to 4 hours.

Benadryl in a 30 mg. dose produced complete protection, but made the animals agitated, restless and unsteady. In a dose of 10 mg. (15 to 17 mg. per kg.) toxic signs were absent, but the protection was not complete, the blue wheal being reduced to a minimum of 3 to 4 mm. in about half an hour.

The maximum non-toxic dose of anthisan which produced complete skin protection against histamine for 3 to 4 hours was between 12·5 and 25 mg., the latter dose producing slight restlessness and agitation. A dose of 17·5 mg. (25 to 30 mg. per kg.) was non-toxic and was equally effective over the same period.

During these tests, a blue staining 1 to 2 mm. round the puncture site occurred after some histamine tests, even during a period of apparently complete anti-histamine protection. As similar "needle-track reactions" often occurred in both anti-histaminized and control animals after any needle puncture, the phenomenon was regarded as unrelated to the histamine injection and probably of mechanical origin.

On the basis of these results antistin in a dosage of 50 mg. per guinea-pig was used in Experiments 1 and 2, 10 mg. of benadryl in Experiments 3, and 17·5 mg. of anthisan in Experiment 4.

Control of anti-histamine action in main experiments.

In Experiments 1, 3 and 4, a guinea-pig which was not burned was introduced into each experiment in order to determine the efficiency of the anti-histamine drug given to the other animals. Evans Blue was injected intracardially, then a histamine skin test was performed and 10 to 20 minutes later the appropriate anti-histamine drug was injected intramuscularly in the same dosage as used in the burned animals. Histamine skin tests were then performed at 1/2 to 1 hourly intervals.

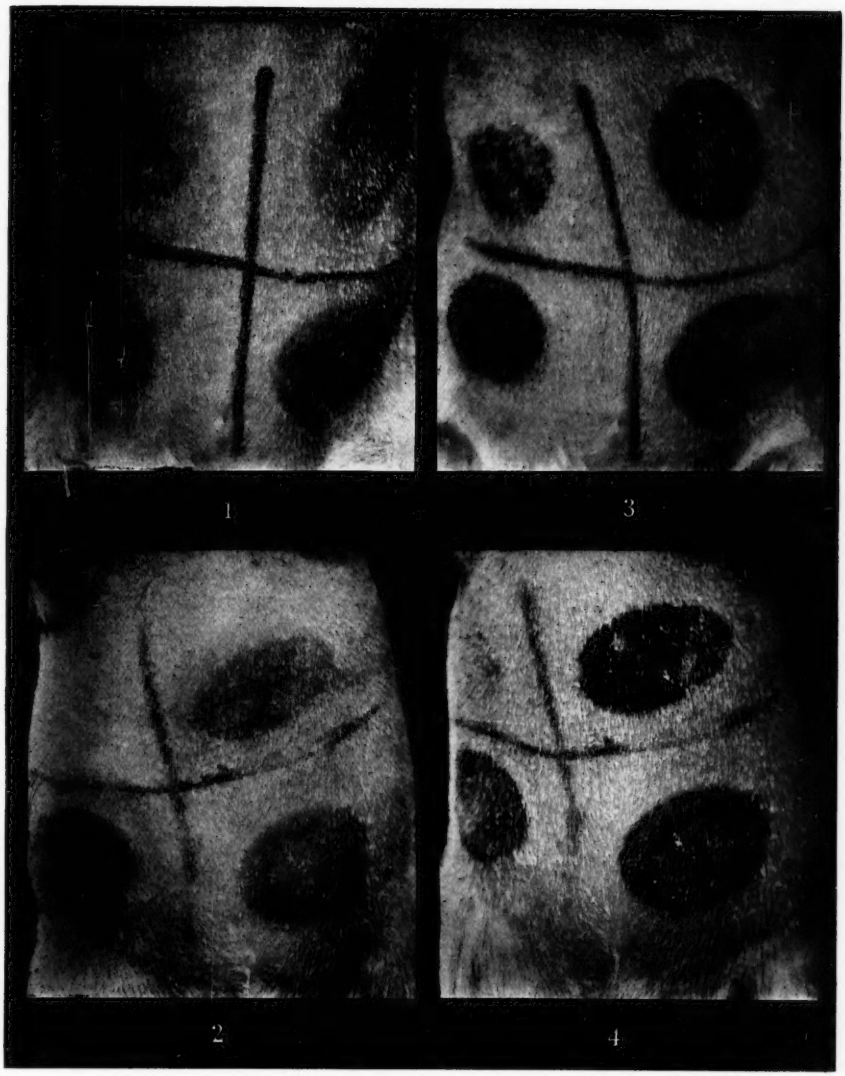
In Experiments 1 and 4 these were completely or almost completely negative for 4 to 5 hours; in Experiment 3, the blue wheal was reduced from 11 mm. in

EXPLANATION OF PLATES.

FIG. 1.—Control animal (C_1). Brilliant Vital Red injected before burning.
FIG. 2.—Anthisanised animal (A_1). Brilliant Vital Red injected before burning.
FIG. 3.—Control animal (C_1). Evans Blue injected 3 hours later.
FIG. 4.—Anthisanised animal (A_1). Evans Blue injected 3 hours later.

FIGS. 1–4.—(See text, Experiment 4) show the similar capillary permeability and blood flow changes occurring in four similar burns inflicted on the abdomens of two guinea pigs, one anthisanised, A_1, the other a control animal, C_1.

Note: The contrast between the red and blue colours on the skin is not so marked in the black and white reproduction as it was *in vivo*.



Sevitt.

diameter before injection of benadryl to a minimum of 5 to 4 mm., the partial protection lasting for longer than 3 hours. In Experiment 2, the " anti-histamine control " animal was not used, but the initial 50 mg. of antistin was supplemented by an additional injection of 12·5 mg. half an hour after burning.

In Experiments 1, 2 and 3 skin histamine tests on all the burned animals were carried out at various times after burning with the intention of directly determining the efficiency of the anti-histamine action in the burned, anti-histaminized animals. Unexpected difficulties in interpretation arose. All the anti-histaminized animals gave the expected weak or negative reactions, and most of the control guinea-pigs showed repeated positive skin reactions, but unexpected weak or negative results were obtained in three of these animals. For example, in one *unprotected* animal a skin histamine test produced a well marked wheal soon after burning ; 1 and 2 hours later similar tests were negative, but after another 2 hours were again positive. In other words, this burned animal became temporarily insensitive to histamine. This result is difficult to explain. However, whatever the explanation of this phenomenon is, it was concluded that when negative skin histamine tests occurred after burning an anti-histaminized animal, the failure of the skin to respond to injected histamine was not necessarily due to the action of the injected anti-histamine drug.

Nevertheless, as a result of the histamine tests on the *unburned* anti-histaminized animals, we may conclude that in Experiment 1, 2 and 4, the burned anti-histaminized animals were completely protected against the effects of a relatively large dose of histamine and that in Experiment 3 the protection was partial.

MAIN EXPERIMENTS.

Experiments 1, 2 and 3.

The general plan of Experiments 1, 2 and 3 is shown in the Table I. At least four animals, A, B, C and D, were used in addition to the unburned " anti-histamine

TABLE I.—*Showing the General Plan of Experiments* 1, 2 *and* 3.

Order of technique used.	Guinea-pig. A.*	B.*	C.	D.	Unburned control.
Evans Blue injected intracardially	+	+	−	−	+
Anti-histamine drug injected intramuscularly	+	−	+	−	+
Repeated histamine skin tests	..	..	..	..	+
Animals burned	+	+	+	+	−
			interval	interval	
Evans Blue injected intracardially	−	−	+	+	−

* In Experiment 2 duplicate animals used.

control " previously mentioned. In Experiment 2, duplicate animals A and B were used. A and B were injected with Evans Blue, and a few minutes later A and C received the appropriate anti-histamine drug intramuscularly. In Experiments 1 and 2, histamine skin tests were performed on animal A 1/2 to 1 hour later and produced no reactions.

Similar burns were then performed on corresponding parts of the backs or abdomens of all the animals. At repeated intervals a clinical estimate of the burn oedema was made (0, +, ++ or +++) and skin sensation tests were performed. In A and B the changes in the burns to circulating dyed plasma was noted, and in C and D the presence or absence of cyanosis in the burn erythema was looked for. Such peripheral cyanosis indicates local slowing of the blood stream (Lundsgaard and Van Slyke, 1923). At definite times after burning Evans Blue was injected into animals C and D, these intervals being 3, 3/4 and 1 hours in Experiments 1, 2 and 3 respectively. The reactions of the burns to plasma dyed at this stage were noted. Most of the animals survived and the depth of skin loss in each burn was clinically determined.

Choice of burns.—On the bases of previous experience (Sevitt, 1949) the temperature and duration of the burns inflicted were so selected as to give a complete range of severity of burning up to, but not including burns which produced immediate heat necrosis of the whole skin. The burns ranged in severity from those in which the capillary permeability changes and subsequent skin loss were absent or slight and in which sensation was unimpaired, to those in which rapid stasis of the local blood flow and analgesia occurred and which were followed by whole thickness sloughing of the skin.

In Experiment 1, four burns at 56° C. and four at 62° C. for 10, 20, 30 and 60 seconds respectively were performed on each animal: In Experiment 2, only two burns at 55° C. for 20 and 30 seconds were inflicted, and in Experiment 3 the burns in increasing order of severity were 54° C. for 20 seconds, 58° C. for 10 seconds and 60° C. for 20, 30 and 60 seconds.

Experiment 4.

In this experiment anthisan (17·5 mg. per animal) was the anti-histamine drug used; the permeability and blood flow changes in the burns were determined by the double-dye technique.

Five albino guinea-pigs were used, one of which was not burned and served as the "anti-histamine control." The other four (A_1, A_2, C_1 and C_2) were paired off, each pair receiving identical treatment, that is to say, each set of observations was duplicated. All were injected intracardially with Brilliant Vital Red: A_1 and A_2 then received intramuscular injections of anthisan, C_1 and C_2 serving as the controls. Half an hour later a series of six burns, all at 61° C., were inflicted on each animal. The severity was varied by using different burning times; burns of 5, 10, 20 and 30 seconds' duration were made on the abdomens, and burns of 45 and 60 seconds were made on the backs. Three hours after burning Evans Blue was injected into all four animals.

RESULTS.

In none of the experiments was any significant difference noted between comparable burns on the control and anti-histaminized animals. In particular no significant differences were observed either in the erythema, capillary permeability or blood flow changes in the burns, in the degree of oedema occurring, in the sensory changes or in the subsequent depths of skin loss. For this reason only the results of Experiment 4 are recorded in detail.

Results of Experiment 4.

The reactions of the abdominal burns in one of the control and one of the anthisanized animals (C_1 and A_1 respectively) are shown photographically in Fig. 1 to 4, where the upper left, upper right, lower left and lower right burns were of 5, 10, 20 and 30 seconds' duration respectively.

In Fig. 1 and 2, which were taken 1 hour after burning, the reactions of the burns to the presence of circulating red-dyed plasma are shown. All the burns in all the animals developed patch reactions, which indicated that the local blood flow was continuing in each burn and was passing through minute dermal vessels of increased permeability. In the 5 seconds' burns, however, the patch reactions (and hence the permeability change) was delayed and less marked than in the remainder. Oedema was not detected in any of the 5 seconds' burns, was very slight or absent in the 10 seconds' burns, but was well marked in all the burns of longer duration. No significant differences were noted, burn for burn, between the control and anthisanized animals during this period.

The reactions of the abdominal burns in Animals A_1 and C_1 to the Evans Blue injected 3 hours later are shown photographically in Fig. 3 and 4. In A_1 a blue patch failed to appear in the 5 seconds' burns, the slight red patch remaining unchanged. This indicated that the slightly increased permeability change of the dermal vessels detected 3 hours previously had now disappeared. In animals A_2, C_1 and C_2, however, blue patches appeared, but were not prominent (Top left burn in Fig. 3). On the other hand, definite and well marked blue patch reactions occurred in all the 10 seconds' burns (Top right burns in Fig. 3 and 4), indicating the continued circulation of the local blood flow and increased capillary permeability change in each of these burns for a period of three hours after burning. In all the 20 seconds' burns, blue rings appeared at the periphery of each red patch, but within half an hour Evans Blue was entering and obscuring each of these red-dyed areas (Lower left burn in Fig. 3). These reactions are of the same nature as temporary ring-with-erythema reactions and indicate extreme retardation or stagnation of the local blood flow. In all the 30, 45 and 60 seconds' burns blue peripheral rings also appeared, but these remained unchanged. Stasis of the dermal blood flow in each burn had occurred.

No real differences were noted, therefore, in the permeability and blood flow changes occurring, burn for burn, between the control and anthisanized animals. Skin sensation was normal in the 5 and 10 seconds' burns. In the 20 seconds' burns sensibility varied with time, that is to say, normal or partial sensibility was present soon after burning, but within a few hours partial or complete analgesia developed. In the 30, 45 and 60 seconds' burns, analgesia was complete within an hour or two of burning. The sensory differences were no more marked burn for burn, between the control and anthisanized animals, than between the two controls or between the anthisanized animals themselves.

Three of the animals (A_2, C_1 and C_2) survived. The degree of skin loss in the 5 seconds' burns was very slight in each burn ; the skin loss was superficial in all the 10 seconds' burns; and in the 20, 30, 45 and 60 seconds' burns whole skin loss developed in all three animals.

CONCLUSION.

Within the limitations of the experimental methods used, the protection of guinea-pigs against histamine by the drugs benadryl, antistin or anthisan has no

detectable effect on the changes produced by burns of different degrees of severity. These changes include erythema of the skin, the presence or absence of permeability change developing soon after burning or present some time later, the development of stagnation or stasis in the dermal blood flow, and the degree of oedema formation. In addition, retention of loss of sensibility to a painful stimulus was not affected nor was the subsequent clinical course of the burn.

DISCUSSION.

The symptoms of anaphylaxis and related disorders, and the local reactions of the body to trauma were explained by Dale (1929), and Lewis (1927) as being due to the liberation of intracellular histamine. In particular, Lewis postulated that when the skin was injured in various ways H-substance was released from the epithelium and acted upon the underlying minute vessels in the dermis. In this way dilatation of the capillaries and venules was produced and this was followed by the exudation of fluid when sufficient H-substance was liberated.

These theories were simple in concept and had the advantage of explaining a multitude of biological phenomena on the basis of the known pharmacological action of a single chemical compound. Moreover, the histamine concept led to a successful search for drugs capable of opposing the action of histamine, and the discovery of these drugs led to their use in experiments and clinical trials, one of the purposes of which was to test the truth of the histamine hypotheses of Dale and Lewis. The results of these have varied ; on the one hand anti-histamine drugs protect a sensitized animal from many of the symptoms of anaphylaxis (Yonkman, Oppenheimer, Rennick and Pellet, 1947) and there is general agreement that they are effective against urticaria (Schindler, 1946 ; Hunter, 1947 ; Overton, 1948), and against hay fever and other forms of spasmodic rhinitis (Southwell, 1948). It would appear, therefore, that the pharmacological action of liberated histamine plays a dominating part in the production of these conditions.

On the other hand, spasmodic asthma rarely responds to anti-histamine drugs (Southwell, 1948). Moreover, the prophylactic use of anti-histamine drugs is unable to suppress or modify the Arthus phenomenon in horse-serum sensitized rabbits (Last and Loew, 1947 ; Dammin and Bukantz, 1949). Furthermore, the reaction of the rabbit's skin vessels to the intradermal injection of horse-serum, trypsin, snake venom, staphylococcus toxin, tetracaine, iodine and heparin are not influenced by the prophylactic injection of benadryl or neoantergan (Last and Loew, 1947). The experiments now reported have shown that neither benadryl, antistin nor anthisan can inhibit or modify the permeability, vascular flow and other changes in the skin of guinea-pigs subjected to burns of different degrees of severity.

There is evidence, however, that not only is histamine liberated from burned tissues, but new formation of histamine may occur (Dekanski, 1945). It follows, therefore, that the ineffectiveness of anti-histamine drugs in modifying the reactions produced by burning does not mean that histamine is not liberated from or not formed in burned tissues, but means that the problem is a much wider one than discovering in which biological reactions histamine is liberated or excreted. In other words, the finding that histamine is released does not necessarily mean that it is responsible for the reaction present, even when this resembles one of the pharmacological actions of histamine. In anaphylaxis, urticaria and

hay fever it would appear that the internal release of histamine plays a major part, but histamine release cannot be very important in the response of the skin vessels to heat.

Another explanation for vascular changes occurring in burns has been put forward (Cullumbine and Rydon, 1946). This is based on the discovery of leucotaxine in inflammatory exudates by Menkin (1940). Intradermal injection of this substance in high dilution causes an increase of capillary permeability and attracts leucocytes to the affected area. Cullumbine and Rydon have found leucotaxine in the vesicle fluid of heat and mustard burns. The leucotaxine theory fundamentally resembles that of Lewis and is subject to similar criticisms. In the absence of a substance antagonistic to leucotaxine this theory cannot be subjected to a critical trial.

The cause of the vascular changes in burns remains unknown, though it is possible that heat itself is the direct noxious agent.

SUMMARY.

The protection of guinea-pigs against the action of histamine by the drugs benadryl, antistin or anthisan (neoantergan) had no detectable effect on experimental burns of various degrees of severity. The changes investigated included the erythema of burns, the capillary permeability and qualitative blood flow changes in the skin, the clinical degree of oedema formation, the skin sensibility and the subsequent clinical course of the burns.

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THE INTRACELLULAR LOCALIZATION OF β-GLUCURONIDASE.

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It has been suggested that the activity of the enzyme β-glucuronidase reflects the degree of cellular proliferation taking place in a tissue or organ (Levvy, Kerr and Campbell, 1948 ; Kerr, Campbell and Levvy, 1949). Evidence has been presented showing that the enzyme activity of tissue extracts is increased in damaged organs undergoing repair, during liver regeneration subsequent to partial hepatectomy, and in the organs of young mice as compared with adults.

Biochemical assay was necessary in order to obtain a quantitative expression of β-glucuronidase activity, but this gives no indication of the actual site of the enzyme in the cell. The recent publication of two histochemical methods (Friedenwald and Becker 1948), now enables the location of the enzyme to be studied.

EXPERIMENTAL.

The methods used for the histochemical detection and localization of β-glucuronidase were essentially those devised by Friedenwald and Becker (1948). Their technique depends upon the hydrolysis at 37° C. of a conjugated glucuronide by the enzyme in fresh frozen sections of tissues, with either the direct liberation of an insoluble azo dye, or the formation of a precipitate of ferric hydroxyquinoline which is converted into Prussian blue. In both methods microscopically visible substances are deposited at the presumptive site of enzyme activity in the tissue.

(1) "1-*ortho-hydroxyphenylazo-2-naphthol glucuronide*" (Friedenwald and Becker, 1948). The substrate solution was made up by dissolving 50 mg. of the glucuronide in 1 litre 0·1 N acetate buffer at pH 5 with warming. When cool, the solution was filtered. Unfixed frozen sections, between 20–40μ in thickness, were incubated in a water bath at 37° C. in 100 ml. substrate solution, in stoppered vessels of such capacity that the air space above the solution was small.

After incubation, the sections were washed in distilled water, fixed in 10 per cent formalin and 1 per cent acetic acid, and either mounted directly in glycerine jelly, or counterstained with 0·5 per cent methyl green in 0·5 per cent acetic acid and then mounted.

Controls for the specificity of the method consisted of incubating the substrate alone, incubating with inert foreign matter, e.g. paper pulp, and incubating with frozen sections of boiled mouse kidney. In no case did hydrolysis occur, even after 18 hours.

(2) 8-*hydroxyquinoline glucuronide* (Brahm, 1899). The substrate solution was prepared as described by Friedenwald and Becker (1948). After the addition

of 0·03 N ferric chloride, followed by incubation at 37° C. for 2 hours, it was found necessary to carry out a double filtration through Whatman No. 40 paper to remove the very fine suspension of ferric hydroxyquinoline which formed. Filtration was carried out in an incubator saturated with water vapour.

It was found advantageous to place the frozen sections in chilled 0·1 N acetate buffer pH 5, as soon as they were cut and to remove as much buffer as possible by touching the sections against a Whatman No. 3 paper before transferring them to the substrate. Small weighing bottles capable of holding about 6 ml. were used as incubation vessels. These had the advantage that there was only a small air space between the liquid surface and the stopper.

The substrate was used on the day it was prepared, and sections were placed in it within 2 to 3 minutes of cutting. One of the main difficulties associated with this method was to obtain a complete conversion of the enzymatically liberated ferric hydroxyquinoline to Prussian blue. This was largely overcome by increasing the concentration of potassium ferrocyanide from 1 per cent to 2 per cent in the ferrocyanide-hydrochloric acid mixture. Sections were fixed as in method (1) and counter-stained with basic fuchsin 1 : 5000 in 1 per cent acetic acid.

RESULTS.

Location of the enzyme.

Fig. 1 shows that the enzyme is localized to the cortical region of the male mouse kidney, and Fig. 2 is an adjacent section incubated in a control solution in which 8-hydroxyquinoline glucuronide was replaced by an equal volume of buffer. A higher magnification (Fig. 3) shows the enzyme to be mainly localized to the proximal convoluted tubules. In suitably thin sections at a still higher power, the enzyme appears to be localized in the basal parts of the cells constituting the proximal convoluted tubules, coincident with the parallel rods frequently to be seen in ordinary histological preparations. These rods are thought to be mitochondria (Maximow and Bloom, 1948). The distal convoluted tubules do not show such an intense reaction, and there is only a very slight staining of Bowman's capsule and the glomerulus of the male kidney (Fig. 5 and 6). Similar localization to the mitochondria was seen in frozen sections of liver (Fig. 7).

Paraffin sections of tissue fixed according to the Gomori technique for phosphatase, showed only a very faint staining with " 1-ortho-hydroxyphenylazo-2-naphthol glucuronide," and none at all with 8-hydroxyquinoline glucuronide.

Influence of pH.

In a series of trials with both glucuronides in which the pH of the substrate was respectively 4·5, 5, 5·2 and 5·5, the best results were obtained within the range 5–5·2 using the normal male mouse kidney.

Inhibition by saccharic acid.

Saccharic acid is known to be an effective inhibitor for β-glucuronidase *in vitro* (Karunairatnam and Levvy, 1949). Its action was utilized to test the specificity of the histochemical methods. Potassium hydrogen saccharate was dissolved in water to give a concentration of 0·1 M. Before final adjustment

of the volume the pH was adjusted to 5 (glass electrode). This solution was added during preparation of either substrate solution in place of part of the acetate buffer.

Using 8-hydroxyquinoline glucuronide as substrate, 0·001 M saccharate caused almost complete inhibition of hydrolysis by a section of male mouse kidney (Fig. 4). Fig. 4 should be compared with Fig. 3, which shows another section from the same kidney, incubated with substrate in absence of saccharate.

To see whether saccharic acid penetrates intact cells, a bisected mouse kidney was incubated overnight in 8-hydroxyquinoline glucuronide solution containing 0·001 M saccharate. As control, the other kidney, also bisected, was incubated in substrate alone. Serial frozen sections from the control kidney showed penetration of the glucuronide to a depth of 20 to 30 cells. Inhibition by saccharate was almost complete at all depths.

Using " 1-ortho-hydroxyphenylazo-2-naphthol glucuronide " as substrate, inhibition by saccharate was not pronounced, although the inhibitor appeared to delay the reaction. A more pronounced inhibition resulted when sections were incubated with 0·001 M saccharate before placing in the substrate and inhibitor. With this glucuronide it appears that the insolubility of the reaction product is so great that the reaction is governed more by the rate of diffusion of the substrate than by the amount of enzyme available. After 2 or 3 hours incubation the fullest possible intensity of staining is seen, independently of the activity of the enzyme present. At shorter periods, some discrimination in intensity is possible between different preparations, but the reaction is not very suitable for comparative work.

Influence of sex on results.

It was incidentally observed that male mouse kidneys gave a more intense staining reaction to both methods than female organs. A suitably short period

EXPLANATION OF PLATES.

(All photographs prepared from frozen sections.)

PLATE I.

FIG. 1.—Longitudinal section of male mouse kidney showing localization of β-glucuronidase to the cortex. Substrate: 8-hydroxyquinoline glucuronide. Incubation time 12 hours. × 12.

FIG. 2.—Control section incubated for the same time in the absence of the glucuronide. × 12.

FIG. 3.—Localization of the enzyme mainly to the proximal convoluted tubules of the kidney. Substrate: 8-hydroxyquinoline glucuronide. Incubation time 12 hours. × 36.

FIG. 4.—Succeeding section to that shown in Fig. 3. Hydrolysis has been inhibited by 0·001 M saccharate. × 36.

PLATE II.

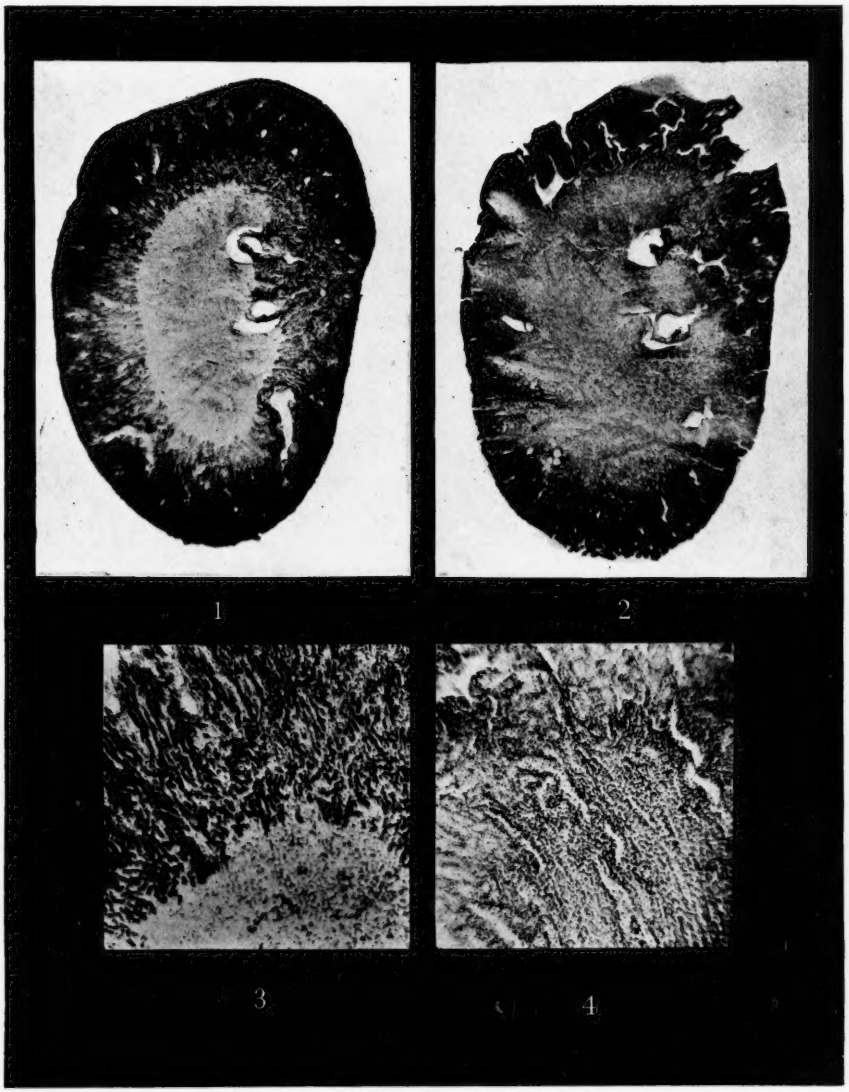
FIG. 5.—Proximal convoluted tubules of the kidney, showing localization of staining to the mitochondria. Substrate: " 1-ortho-hydroxyphenylazo-2-naphthol glucuronide." Incubation time 80 minutes. × 300.

FIG. 6.—As for Fig. 5. The glomerulus is unstained. Incubation time 160 minutes. × 300.

FIG. 7.—Specific staining of the mitochondria of mouse liver cells. Substrate: " 1-ortho-hydroxyphenylazo-2-naphthol glucuronide." Incubation time 80 minutes. × 300.

FIG. 8.—3:4 benzpyrene induced squamous cell carcinoma of skin of mouse, showing intense staining of the malignant cells growing down into the corium. Substrate: 8-hydroxyquinoline glucuronide. Incubation time 15 hours. × 36.

FIG. 9.—Granular and filamentous mitochondria staining specifically in the G.R.C.H./15 chicken fibro-sarcoma. Substrate: 8-hydroxyquinoline glucuronide. Incubation time 10 hours. × 300.



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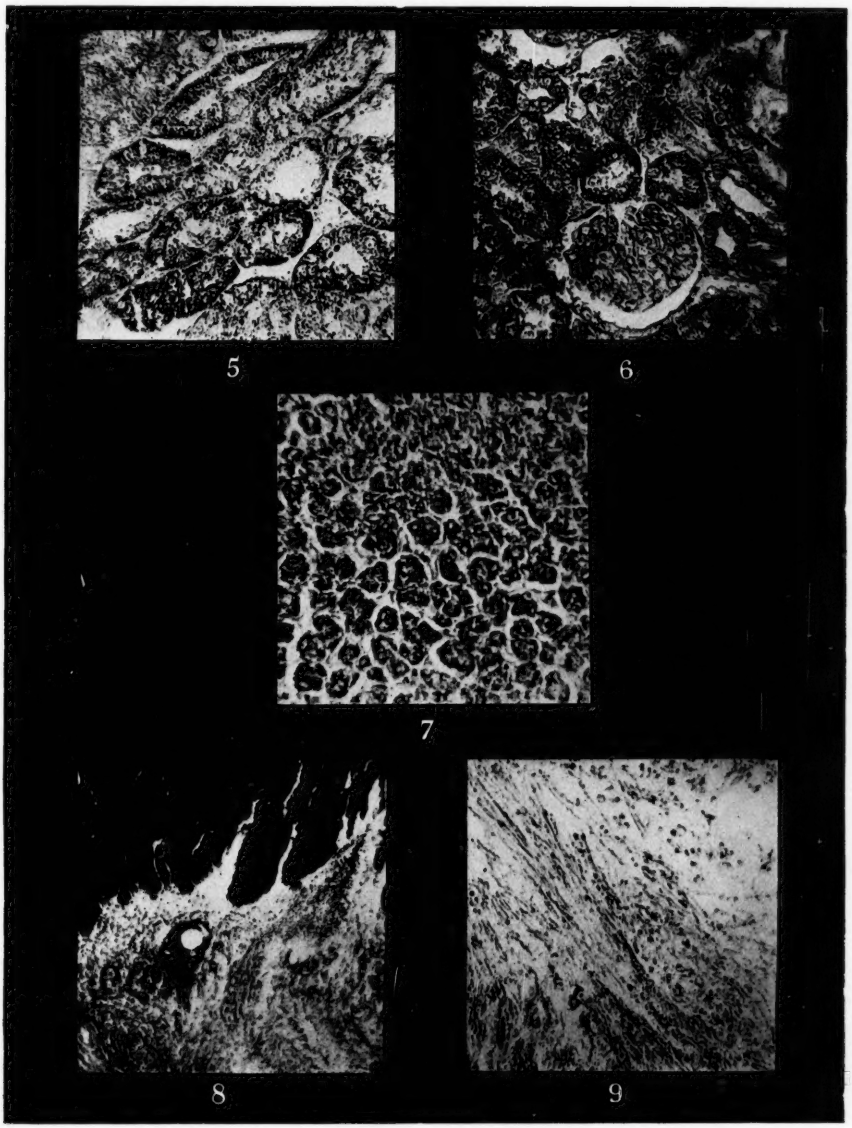

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of incubation was used in Method 1. The hydrolytic reaction was reduced in castrate male mouse kidneys, and increased in kidneys from ovariectomized mice, in both cases 8 weeks subsequent to operation. Localization of the enzyme was identical in male or female kidneys.

Results with tumour tissues.

Great difficulty was experienced in obtaining satisfactory histochemical pictures using rapidly-growing malignant tumours, because of frequent widespread necrotic changes, while in certain growths the presence of large amounts of mucoid material interfered with the reaction. However, although results were often unsatisfactory from the photographic aspect, a fair picture of the distribution of the enzyme was obtained in a preliminary study. As a whole, tumours seemed high in enzyme activity compared with normal liver and kidney. Table I summarizes the findings in those tumours examined.

TABLE I.

Tumour.	Enzyme distribution.
Rous sarcoma, fowl	Scattered discretely in cytoplasm.
G.R.C.H./15, fowl	,, ,,
Ovarian adenocarcinoma, fowl . .	Traces of enzyme confined to glandular epithelium. None in schirrous areas.
Mouse mammary carcinoma . . .	Rich in anaplastic areas, none in blood cysts, degenerated parts or connective tissue.
Squamous cell carcinoma, skin of mouse .	Rich in poorly differentiated down-growing epithelium. Traces in dermis.
Papilloma, skin of mouse . . .	None.

Assessing these preliminary observations, it may be concluded that β-glucuronidase is most rich in those parts of tumours where cell division is a prominent feature. This is especially well shown in Fig. 8 where a very pronounced staining is practically confined to the down-growing malignant epithelial cells of a mouse skin epithelioma induced by an injection of 3 : 4 benzpyrene. As in normal tissues, suitable preparations show the enzyme to be confined to the cytoplasm of tumour cells (Fig. 9).

DISCUSSION.

Fishman (1940) showed that the prolonged administration of menthol to mice caused a rise in the activity of β-glucuronidase in the liver, kidney and spleen. He postulated that this enzyme acts synthetically, but this has not yet been established and other workers take the view that the function of the enzyme in the animal body is purely hydrolytic (Levvy, 1947 ; Karunairatnam, Kerr and Levvy, 1949.)

In a later report, Fishman and Anlyan (1947) demonstrated a high β-glucuronidase content in some human tumours. It is interesting to note that

in Table I of their communication, the activity of the enzyme was closely correlated with the clinical malignancy of the tumour. For example, benign tumours showed a low activity, tumours such as schirrous carcinoma also low, whilst adenocarcinoma and medullary carcinoma had high enzyme activities. It is difficult in cancer studies to obtain the corresponding normal tissue unless an organ like the liver is involved, and a serious criticism may be made of the normal tissue controls used by Fishman and Anlyan (1947). Whereas an organ may be a complex of tissues, only one of these is usually involved in neoplasia. The fact remains, however, that striking elevations of the enzyme figures occurred in malignant tumours compared with normal tissues or organs from which those tumours arose. This work has been confirmed and extended by Odell and Burt (1949).

Kerr and Levvy (1947) at the same time as Fishman's work on tumours, showed that a rise in enzyme activity occurred in mouse liver or kidney following administration of toxic agents not known to form glucuronides, dependent on the site of damage. The action of menthol, which was found to be a liver and kidney poison could be explained on the same terms. Kerr, Levvy and Campbell (1947) found that the rise in the enzyme activity was not detectable in the early stage of poisoning, as, for example, with mercuric nitrate or chloroform in the kidneys of the male mouse, but occurred as soon as repair was histologically demonstrable. Furthermore, sub-total hepatectomy of mice, either by ligation or cautery, caused rapid hypertrophy of the remainder of the organ, a great deal of mitotic activity and an elevation of β-glucuronidase activity. It was also found that the liver, spleen and kidney of baby mice showed much higher enzyme levels than in adults. This was also true of uterus (Kerr, Campbell and Levvy, 1949).

It has been shown by Crabtree (1941) that there is a morphological difference in the cells of Bowman's capsule in male and female adult mice. Eschenbrenner and Miller (1945) demonstrated the specific toxicity of chloroform for the convoluted tubules of the male mouse kidney. Furthermore they showed that the epithelium of Bowman's capsule in castrated male mice tended to change to the female type, and that the kidneys of such mice were no longer susceptible to chloroform poisoning. These findings were confirmed (Kerr, Campbell and Levvy, 1949) and it was also shown that a rise in β-glucuronidase occurred in male kidneys subsequent to the administration of chloroform, but not in female kidneys or kidneys of castrated male mice, and the rise, when demonstrable, was coincident with repair processes in the damaged organ.

Further evidence for a relationship between cell proliferation and β-glucuronidase activity appears to be provided by Fishman and Fishman's (1944) observation that the administration of oestrogens to ovariectomized mice causes a rise in uterine glucuronidase, their explanation being that glucuronidase is primarily concerned with the conjugation of oestrogen. A more likely explanation would be on the basis of proliferating endometrium. The effect can be prevented by the concurrent administration of androgen (Kerr, Campbell and Levvy, 1949). Odell and Burt (1949) consider that the association between enzyme activity and proliferative activity provides the best explanation of their results for human cervical cancer, and those conditions characterized by an increased rate of cell division such as pregnancy and the proliferating corpus luteum.

The first part of the present paper deals with preliminary findings using histochemical methods for the localization of β-glucuronidase. The association with mitochondria is perhaps not unexpected, as it does not seem unreasonable to assume that many intracellular enzymes must be associated with these organoids, which in the living cell are in constant movement, exhibit changes of form, and frequently show a well marked tendency to polarity, all indicative of intracellular activity.

It is significant that the enzyme is confined to that part of the kidney parenchyma, the cortex, which is damaged in chloroform poisoning.

The experiments with saccharate suggested that the test with 8-hydroxyquinoline glucuronide at least, is specific for glucuronidase. It is interesting to note that the experiments give unequivocal evidence of penetration of saccharic acid into the intact cell. The failure of saccharic acid to influence glucuronide synthesis by surviving liver slices (Karunairatnam and Levvy, 1949) thus provides excellent grounds for believing that β-glucuronidase is not involved in the synthesis of glucuronides *in vivo*. In the few histochemical observations made to date and described above, β-glucuronidase appears to be particularly rich in actively growing tumours or parts of tumours.

SUMMARY.

Using techniques devised by Friedenwald and Becker (1948), the localization of β-glucuronidase has been studied in normal mouse kidney and liver, and in several chicken and mouse tumours. In the kidney, enzyme activity is practically confined to the proximal convoluted tubules in the cortex.

The intracellular site of the enzyme in both liver and kidney appears to be coincident with the mitochondria.

The hydrolysis of 8-hydroxyquinoline glucuronide, by fresh frozen mouse kidney sections is inhibited by 0·001 M saccharic acid. Serial frozen sections of a bisected kidney treated in the same manner, show penetration of the cells by saccharic acid, as indicated by inhibition of hydrolysis.

In unit time, the male mouse kidney shows a stronger staining reaction in these histochemical methods, than the corresponding female organ. Castration of males diminishes this effect, whereas ovariectomy augments it, when compared with the enzymatic activity of kidneys from intact animals of the like sex.

In a preliminary study the enzyme activity is found to be high in malignant tumours, and is mainly localized in anaplastic areas where cell division is prominent.

The results obtained by a number of workers can be interpreted in terms of a relationship between β-glucuronidase activity and proliferative activity.

In chemical and biochemical aspects of this work, the author is indebted to Dr. G. A. Levvy for practical assistance and suggestions. Thanks are also due to Mr. G. M. MacKenzie for technical help. The British Empire Cancer Campaign defrayed all expenses.

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THYROID-PARATHYROIDECTOMY IN PARABIOTIC RATS.

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TUMOUR formation in the rat thyroid under the influence of goitrogens has been attributed to the continuous stimulation of the thyroid cells by excessive amounts of thyrotropic hormone (Griesbach, Kennedy and Purves, 1945; Money and Rawson, 1947). It seemed desirable, however, to exclude the possibility that the goitrogenic agents, apart from causing indirectly the release of large amounts of thyrotropic hormone, had a direct carcinogenic effect on the thyroid cells. The induction of thyroid adenomata in the rat under conditions of extreme iodine deficiency, leading to thyrotropic stimulation and consequent hyperplasia of the thyroid, has been considered to have proved the fact that a physiological agent, acting intensively for a long time, can produce neoplastic cell changes. It occurred to us that the method of parabiosis might provide a means of stimulating the thyroid without introducing a foreign chemical agent or subjecting the animal to a state of thyroxine deficiency. It is well known that after castration of one parabiotic partner large amounts of gonadotropic hormone released from the castration pituitary produce marked hyperplasia and hypertrophy of the other partner's gonads. The sex hormones produced by the intact partner are so rapidly destroyed that the amounts transferred to the castrated partner are insufficient to inhibit this animal's production of gonadotropic hormones (Zeckwer, 1946). If a similar situation occurred after thyroidectomy the thyroid of the intact animal would be subjected to a continuous stimulation by the thyrotropic hormone from the thyroidectomized partner. Necessary conditions would be (*a*) rapid destruction or excretion of thyroxine by the intact partner, and (*b*) the passage of sufficient amounts of thyrotropic hormone across the junction from the thyroidectomized animal. Naiko and Ikonen (1934), in rats, did not find any changes in the thyroid when parabiosis between a normal and a thyroidectomized animal was performed. This result has been confirmed and, in addition, changes in the pituitary and parathyroid are described.

METHODS.

The animals used were rats of a Wistar strain, 6 to 10 weeks old. Litter mates of the same sex were joined in parabiosis, with open abdominal junction, and three weeks later the right partner of pairs which had shown satisfactory growth was thyroidectomized.

The diet fed was free of meat protein, and has been described in a previous publication (Diet S3, Griesbach, Kennedy and Purves, 1945). The iodine content of this diet was low and a number of pairs was supplied with additional iodine in the drinking water (1 μg. I_2 as KI per ml.). One pair (No. 1) received no additional iodine; pairs 6 to 11 were provided with approximately 10 μg. daily

throughout the experiment, and pairs 2 to 5 received this supplement during the first weeks, but it was withdrawn for the last 104 days.

The animals were killed 15 to 233 days after thyroidectomy. The pituitaries were fixed in sublimate formalin, and stained according to Martin's modification of Mallory's method. The thyroids were fixed in formol saline and the cell heights measured as described by Griesbach and Purves (1943).

RESULTS.

Growth.

In ten of the eleven pairs the growth of the thyroidectomized animal was comparable with that of the intact partner. The maximum weight reached by a pair was 444 g.

Thyroids.

The weights and mean acinar cell heights of thyroids of pairs 6 to 11 which received iodide throughout the experiment were normal (10 mg. per 100 g. and 7·1μ). In the group which received additional iodide during the early part of the experiment only, the weights and mean cell heights were increased (average 13·4 mg. per 100 g. and 10·1μ). The thyroid of pair No. 1 was enlarged (28·4 mg. per 100 g.).

Pituitaries.

(1) Thyroidectomized partners (R): Acidophil cells were present in all pituitaries, but in slightly lower numbers than in our normal animals, which have an average value of 48 per cent. In ten animals the number of basophil cells was high and typical thyroidectomy changes were present. One rat, No. 5, did not have an increased number of basophils although no thyroid could be found at autopsy.

(2) Intact partners (L): The pituitaries of these partners showed well-granulated acidophil cells, the percentage of which was higher in the group receiving a continuous iodide supplement. Rat 1, which received no additional iodide had a definite basophilia, Rat 2 a slight basophilia, while the others showed normal figures. These results are summarized in Table I.

Gonads.

The two pairs of female rats had normal oestrous cycles. Both partners gave birth to normal litters.

Parathyroids.

The parathyroids of all the intact partners were enlarged. Microscopically, they showed a high activity: The cells were enlarged, the nuclei were vesicular and poor in chromatine: there was an abundance of clear cells with eccentric nuclei. The dark cells were scarce and were found only at the periphery (Fig. 1 and 2).

The blood calcium content examined in five pairs was not consistently higher in one partner. The values ranged from 7·4 to 13·1 mg. per cent. No bone changes were found histologically.

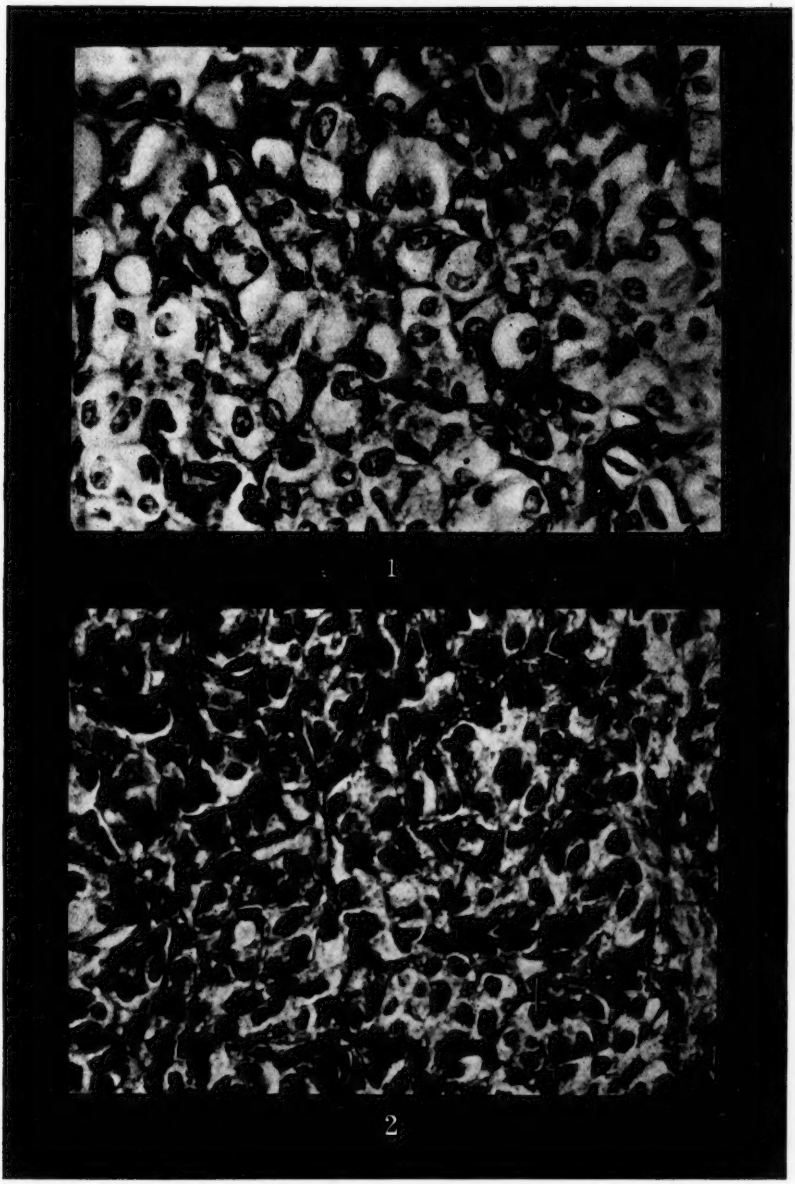

FIG. 1.—Parathyroid of intact partner (L) after 233 days of parabiosis. Majority of cells represents the water clear type. × 650. H. & E.
FIG. 2.—Parathyroid of normal rat of same age. × 650. H. & E.

TABLE I.—*Thyroid and Pituitaries of Parabiotic Rats after Thyroidectomy of the Right Partner (R).*

No.	Sex.	Days after thyroidectomy.	Body wt. (g.) (L).	Body wt. (g.) (R).	Thyroid wt. (mg. %).	Cell height (μ).	Pituitary cells (%). L. Acid.	Pituitary cells (%). Bas.	Pituitary cells (%). R. Acid.	Pituitary cells (%). Bas.
1	M.	55	210	150	28·4	9·3	50·2	22·9	18·5	34·4
2	M.	202	230	214	12·6	12·6	61·2	11·5	46·6	29·7
3	M.	233	170	163	17	9·5	56·2	7·6	39·7	37·1
4	F.	233	165	179	14·5	10·3	56·8	6·3	39·3	27·9
5	F.	233	164	184	9·1	9·9	49·5	5	40·4	9·3
*6	M.	15	83	75	<10	7·6	47·2	5·1	48·9	23·4
7	M.	15	149	114	<10	5·9	50·3	4·6	36·8	30·7
8	M.	26	108	70	<10	6·8	44·9	9·2	40·8	28·6
9	M.	100	146	127	10·9	7·1	48·1	7·3	32·9	40·9
10	M.	100	124	173	11·4	6·9	47·5	6·2	40·9	38·4
11	M.	130	159	128	<10	8·1	48·8	3·8	43·8	26·6

* No. 6 to 11 received 0·1 mg. per cent. KI in the drinking water.
No. 1 to 5 did not get KI in their drinking water during the last 104 days.

DISCUSSION.

The close correlation between the numbers of "thyroidectomy cells" and the grade of thyroxine deficiency has been described in an earlier paper (Griesbach and Purves, 1945). The results reported in this paper suggest a considerable thyroxine deficiency in the thyroidectomized partners (R). Ten of the eleven pituitaries showed a high percentage of basophils (23 to 41 per cent). It is reasonable to assume, therefore, that these pituitaries were secreting abnormal amounts of thyrotropin into the blood (Griesbach and Purves, 1943). The thyroxine deficiency in the R-animals was not absolute, however, for their pituitaries contained a normal percentage of well-granulated acidophil cells, with the exception of No. 1. Such a cytological picture would suggest that an amount equivalent to at least 1 μg. of DL-thyroxine per 100 g. of body weight per day (Purves and Griesbach, 1946) had passed from the intact to the thyroidectomized animal. In one instance (Rat 5) the amount of thyroxine that had been transferred must have approached the daily requirement dose of 2·3 μg. DL-thyroxine per 100 g. of body weight, for the pituitary of the animal showed a cell distribution approximating the norm. In this animal no thyroid was found at autopsy.

The assumption that thyroxine passed over the junction is supported by the growth of the thyroidectomized animals and the fact that thyroidectomized females underwent normal pregnancy.

In the groups receiving additional iodide (No. 6 to 11) the thyroids of the intact partners showed normal weights and epithelial cell heights. This result was obtained in animals sacrificed from 15 to 130 days after thyroidectomy of the one partner. Apparently the blood thyrotropin level in the intact animal had not been sufficiently high to produce any marked stimulation of the thyroid.

In the group of rats which did not receive additional iodide a slight enlargement of the thyroids was found in some animals. The thyroid of pair No. 1,

which had not received any iodide supplement, was enlarged and showed hyperplastic epithelium. The basophilia (22·9 per cent) in this animal's pituitary is sufficient to account for the thyroid stimulation observed and is probably due to an iodine deficiency. The low thyroxine production in this pair was further evident from the small percentage of acidophil cells in the thyroidectomized partner's pituitary.

Pairs No. 2 to 5 had been given iodide for 4 months after thyroidectomy, but this had been withdrawn during the last 3½ months. The thyroid of pair No. 5, while not enlarged, showed increased acinar cell heights and had apparently supplied the partner R with enough thyroxine to maintain a nearly normal pituitary. The remaining three pairs showed slight enlargement of the thyroids with activated epithelium. No conclusion can be drawn from these experiments as to the source of the thyrotropic hormone responsible for this slight activation. The hormone may be derived from the animal's own pituitary or transferred from the thyroidectomized partner. This problem could be approached by parabiotic union of a thyroidectomized with a hypophysectomized animal. Du Shane, Levine, Pfeiffer and Witschi (1935), who combined hypophysectomized with normal or castrated rats, could not observe any thyrotropic effect on the hypophysectomy thyroid, and concluded that insufficient thyrotropin is transferred through the junction. The older experiments of Naiko and Ikonen (1934), and those presented in this paper, lead to the conclusion that even the large amounts of thyrotropin released by the thyroidectomy pituitary were not exerting any conspicuous action on the partner's thyroid.

The enlargement of the parathyroids was quite striking. The cells appeared to be in a state of high activity, with a predominance of clear cells. This parathyroid enlargement and the cell changes were observed in all the intact animals. Anselmino, Hoffmann and Herold (1934) described changes in rat parathyroids following injections of anterior pituitary extracts. The changes seen in our animals resembled closely those described by these authors, and suggested increased activity of the glands. This could be explained by the assumption that a parathyrotropic factor is transferred from the parathyroidectomized animal. The existence of a pituitary parathyrotropic hormone has been questioned, however, by other workers.

SUMMARY.

Thyroid-parathyroidectomy was carried out in one partner of parabiotically joined rats.

The thyroids of the intact partners did not show any marked stimulation. The amount of thyrotropic hormone passing from the thyroidectomized to the intact partner must therefore be negligible.

Some thyroxine (equivalent to about 1 μg. DL per 100 g. per day) was transferred from the intact to the thyroidectomized partner.

The parathyroids were enlarged and consisted predominantly of water clear cells.

Our thanks are due to Dr. D. C. Van Dyke, Berkeley, Cal., for his instruction on the technique of parabiosis, and to Mr. R. Malthus, B.Sc., of the Nutrition Research Department, for the calcium determinations.

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THE EFFECT OF SODIUM SALICYLATE UPON HYPERSENSITIVITY REACTIONS.

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SALICYLATE treatment of rheumatic fever is based both on clinical observation and experimental work. Its palliative effect is generally accepted, but, in addition, there is a considerable weight of evidence that it may arrest some of the pathological changes of the disease (Coburn, 1945; Reid, 1948; Small, Wissler and Watson, 1948). Whilst the etiology of rheumatic fever is still an unsolved problem there is little doubt that the vascular endothelium is involved from the earliest phases and that the lesions invite comparison with those resulting from antigen-antibody reactions of the hypersensitive or allergic type. Indeed, much recent work supports the hypothesis that hypersensitivity, following upon infection with haemolytic streptococci, lies at the root of the rheumatic process. Provisionally accepting this hypothesis it seemed to us that the effects of salicylate therapy might depend upon interference with hypersensitivity reactions and that, therefore, a study of the effects of salicylates on such reactions, experimentally induced in animals, might throw some light upon rheumatic fever pathogenesis. During the course of the work Swyer (1948) published observations to the effect that in rabbits salicylates prevent the increased capillary permeability induced by intracutaneous injections of histamine. We therefore extended our investigation to include experiments with certain anti-histamine drugs. Swyer's claim was readily confirmed but the experiments revealed interesting differences between the actions of these anti-histamine drugs and of sodium salicylate. The drugs used were "Antistin" (2-phenyl-benzylamino methyl imidazoline methane sulphonate); "Anthisan" (N-dimethylamino ethyl-N-*p*-methoxy-benzyl amino pyridine maleate); "Phenergan" (N-(2-dimethylamino-m-propyl) phenothiazine HCl). Throughout this paper they are referred to under their proprietary names.

EXPERIMENTAL.

Salicylate Treatment of Experimental Animals.

Freshly prepared, Seitz filtered, aqueous solutions of sodium salicylate, B.P. standard, were used. For intravenous and intraperitoneal administration the solutions were made approximately isotonic with 0·9 per cent NaCl (2·5 to 3 g. per 100 ml.) but for oral administration 5 per cent to 10 per cent solutions were used in order to reduce the volumes required.

* Attached to the department as a member of the external staff of the Medical Research Council.

Our aim was to maintain the blood salicylate concentrations of treated animals, throughout the period of experimental observation, at levels comparable with those attained in human patients undergoing intensive salicylate therapy. The doses chosen for different experiments were therefore based on a preliminary investigation of excretion rates in guinea-pigs and rabbits, the relevant results of which are summarized in Table I. Rabbits showed much greater variation than guinea-pigs in excretion rates. All the doses in guinea-pigs, and some in rabbits, produced hyperpnoea but otherwise were without deleterious effect. Plasma salicylate concentrations were estimated by the method of Brodie, Undenfriend and Coburn (1944).

TABLE I.—*Plasma Salicylate Levels in Guinea-pigs and Rabbits.*

Species.	Sodium salicylate.		Plasma salicylate concentration.	
	Route.	Dose g. per kg.	mg. per 100 ml.	Duration (hours).
Guinea-pigs	Intraperitoneal	0·4	45–80	4
			> 25	6
	Subcutaneous	0·4	40–60	3
	,,	0·6	50–80	6
	Oral*	0·55	50–80	6
Rabbits	Intraperitoneal	0·4	> 50	4
			> 30	6
	Intravenous	0·1	> 30	1
	,,	0·2	> 40	2

* Given by stomach tube.

The Effect of Sodium Salicylate on Anaphylactic Shock in Guinea-pigs.

Guinea-pigs were either actively sensitized by intraperitoneal inoculation of 0·01 ml. horse serum in a volume of 2 ml. or passively sensitized by intracardiac inoculation of a pooled rabbit serum from animals which had been immunized against bovine serum albumen. A shock dose of the antigen was used which could be expected to kill approximately 50 per cent of the control animals; it was diluted in saline to a volume of 0·25 ml. and inoculated intracardially into the right ventricle, except in one experiment in which each animal received a volume of 0·5 ml. intraperitoneally. The anaphylactic nature of deaths which occurred was confirmed by autopsy examination.

Sodium salicylate was administered to the test animals from half to 2 hours before the shock dose of antigen and, at the same time, control guinea-pigs, matched as regards weight, were given corresponding quantities of NaCl solution. When the antigen was injected intracardially the dose of sodium salicylate was 0·4 g. per kg. intraperitoneally or, in one experiment, 0·7 g. per kg. orally. When the antigen was injected intraperitoneally 0·6 g. per kg. sodium salicylate was given subcutaneously. Thus, in all experiments the plasma level of sodium salicylate was over 40 mg. per 100 ml. at the time of test (see Table I).

A running commentary on each guinea-pig was recorded verbatim by a stenographer from the moment of injection up to the time of death or the onset

of recovery. From this record the degree of shock was assessed and the animal was given a shock score as follows :

Trivial symptoms	0·5
Mild shock	1
Severe shock	2
Very severe shock with eventual recovery	3
Death after more than 10 min.	4
Death within 10 min.	5

The results are summarized in Table II. They were closely similar with both actively and passively sensitized guinea-pigs. There was no clear-cut evidence of protection by salicylate treatment, but there was a tendency for treated animals to survive a little longer and to exhibit slightly less severe symptoms than controls. The difference appeared rather more striking during actual observation than the small score differences would suggest.

TABLE II.—*Effect of Sodium Salicylate on Anaphylactic Shock in Guinea-pigs.*

Sensitization.	Shock dose of antigen.	Number of guinea-pigs.		Average shock scores*	
		S.	C.	S.	C.
Active with Horse Serum	0·01 ml. intracardiac	10	11	3·5	4·3
	1–5 ml. intraperitoneal	6	6	3·7	3·7
Passive with anti-bovine albumin rabbit serum	0·4–1 mg. antibody N.	6	6	3·4	4·1

S. = Salicylate treated animals.
C. = Untreated controls.
* Shock scores.—(For explanation see text.)

As a check upon the suitability of our experimental technique three actively sensitized guinea-pigs were each given 20 mg. per kg. Antistin intramuscularly and the shock dose of horse serum was inoculated intracardially 30 min. later. None of the animals showed any sign of acute anaphylaxis, but one to three hours later they were all mildly prostrated with ruffled fur. This supports the contention that although Antistin can suppress completely the immediate manifestations of anaphylactic shock, it may fail to prevent later prostration, which is possibly caused by factors other than histamine (Rosenthal and Brown, 1940 ; Rose, 1947).

Arthus Reactions in Rabbits and Guinea-pigs.

Effect of sodium salicylate upon direct Arthus reactions.

An experiment was made in order to discover whether any clear effect of sodium salicylate could be demonstrated under the relatively uncontrollable conditions of the direct Arthus phenomenon in actively sensitized rabbits. Six animals were sensitized with human sera, and, on the basis of preliminary skin tests, were divided into two groups of as nearly equal sensitivity as possible.

Two days later they were tested by intracutaneous inoculations of 0·1, 0·01, 0·001 and 0·0001 ml. human serum. Rabbits of the group considered to have shown slightly greater sensitivity in the preliminary test were given four intraperitoneal injections of sodium salicylate, 0·4 g. per kg., at eight-hourly intervals whilst those of the control group received corresponding amounts of NaCl. Areas of oedema were measured at 6, 24 and 48 hours. The results showed no significant differences between the groups, although reactions during the first 24 hours appeared to be less intense in the salicylate treated rabbits. Fischel (1947) reported a similar, rather doubtful, early action of salicylates in experiments of this type.

Effect of sodium salicylate upon passive reversed Arthus reactions.

Attention was now turned to the passive, or reversed, Arthus reaction. In a careful study Fischel and Kabat (1947) showed that the intensity of the erythema and oedema in this reaction is proportional to the amount of antibody injected intracutaneously but is independent of the amount of antigen injected intravenously over a wide range. It is thus possible to carry out the reaction quantitatively by inoculating known amounts of antibody 30 min. after the intravenous injection of a suitable amount of antigen. Furthermore, the reaction can be repeated on the same animal provided it is done within two or three days, that is before the injected antigen has had time to elicit any appreciable antibody response. To do this further antigen is given intravenously, and the original dose of antibody injected at another skin site. Residual antigen from the first test does not affect the result since the intensity of reaction is relatively independent of the amount of this reagent in the circulation. Thus each animal can be used successively as a test and control animal or vice versa.

Rabbit experiment.—Six rabbits were inoculated intraperitoneally with 0·4 g. per kg. sodium salicylate, and six were given a corresponding volume of 0·9 per cent NaCl. After 30 min. all received an intravenous injection of bovine serum albumen (2 mg. N. per kg.). After a further 30-min. interval each rabbit was inoculated at two separate skin sites with 0·5 ml. rabbit anti-bovine albumen serum containing 0·48 mg. antibody nitrogen per ml. Further intraperitoneal injections of sodium salicylate and NaCl were given at 7 hours and 15 hours from the start of the experiment. Areas of oedema at skin injection sites were measured at 4 hours and 24 hours and subjective estimates of reaction intensities were made. The result for each animal was taken as the average of the readings of the two sites.

Two days later the roles of the two groups were reversed, rabbits previously treated with sodium salicylate now receiving NaCl and vice versa. The experiment was repeated, except that the quantity of the antigen injected intravenously was reduced to 1 mg. N. per kg. in order to allow for residual circulating antigen.

The results are presented in Table III, but subjective estimates of intensities of the reactions are omitted as they agreed closely with the objective measurements of oedema. There is no doubt that the salicylate treatment, whilst not entirely preventing the development of Arthus reactions, did cause very definite decrease in their size and intensity. If there had been any considerable variation of skin sensitivity in the rabbits between the first test and its repetition two days later the bias would have affected groups A and B oppositely. This was not the

TABLE III.—*Effect of Scdium Salicylate on Reversed Passive Arthus Reactions in Rabbits.*

Six rabbits in each group.	Mean areas of oedema (cm²). Group A.	Group B.	Combined A and B.
When salicylate treated at 4 hr. . . .	3	2·3	2·7
,, ,, 24 ,, . . .	3·7	3	3·4
When given NaCl as Controls at 4 hr. . .	5·4	6	5·8
,, ,, 24 ,, . .	6·6	5·7	6·2

case, for in 11 out of the 12 animals the reactions during their period on salicylates were less at all stages of development than the reactions elicited during their period as control animals. The remaining animal was ill during the control period, which may have affected its skin reactivity.

It was observed that small punctate haemorrhages occurred at the sites of the Arthus reactions in 10 out of the 12 rabbits when they were under salicylate treatment, but not when being treated with NaCl. We are unable to offer any explanation of this phenomenon.

Guinea-pig experiment.—A similar experiment was carried out with guinea-pigs. Two groups, each of five guinea-pigs, were used alternately as salicylate treated and untreated animals in duplicate tests. For salicylate treatment a total of 900 mg. per kg. was injected intraperitoneally in three doses at 30 min. before, and 5 and 12 hours after, the intracutaneous inoculation of antibody, saline in corresponding volume being given to the untreated group. The antigen and antibody used were the same as in the rabbit experiment with dosage suitably adjusted for the smaller species. The antigen was given intracardially an hour before the antibody. Inoculation of the rabbit anti-serum into unsensitized guinea-pigs was shown to produce nothing more than a small, transient reaction. Guinea-pigs have seldom been used for experiments involving the Arthus phenomenon, probably because the skin reactions induced are less superficial and hence much less evident upon cursory examination than are the reactions in rabbits. It was indeed found impracticable to compare responses by measurement of surface areas alone, but valid comparisons could be based upon oedema volume calculated by the arbitrarily adopted formula, volume $= \frac{1}{3} \times$ surface area $\times$ depth. Five sets of measurements were made at intervals during the 24-hour period of observation. The results are presented graphically in the figure, mean oedema volumes for the ten animals when salicylate treated and when untreated being plotted against time. The wide separation of the two curves throughout the whole period of observation indicates the striking inhibitory effect of salicylate treatment upon the Arthus reaction in guinea-pigs.

The effect of anti-histamine drugs on passive reversed Arthus reactions.

The striking effect of Antistin in preventing acute anaphylactic shock in guinea-pigs led us to study the effect of this drug upon Arthus reactions in rabbits. The procedure was identical with that employed for the salicylate experiment except that the treated rabbits received three intramuscular injections of 50 mg. Antistin during the first 12 hours, whilst control animals received no treatment

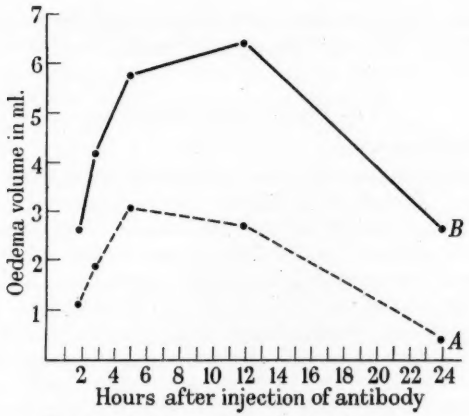

FIG. 1.—Arthus reactions in 10 guinea-pigs.

(*A*)—Mean oedema volume of reactions induced during salicylate treatment.
(*B*)—Mean oedema volume of reactions induced during control period.

at all. Ten rabbits were used, each group of five serving as controls to the other group in one of the two tests. The results are given in Table IV. Reaction differences with and without Antistin are in the same direction as in the salicylate experiment but are very much less marked.

TABLE IV.—*Effect of Antistin on Reversed Passive Arthus Reactions in Rabbits.*

Five rabbits in each group.	Mean areas of oedema (cm²).		
	Group A.	Group B.	Combined A and B.
When Antistin treated at 4–5 hr. . . .	5·6	5·8	5·7
,, ,, 24 ,, . . .	7·8	7·5	7·7
When untreated controls at 4–5 hr. . . .	5·9	8·9	7·4
,, ,, 24 ,, . . .	7·8	8·9	8·3

Both Antistin and Anthisan were also tested in guinea-pig experiments on groups of 8 and 6 animals respectively. In each case the relatively very large amount of 60 mg. of the drug was given intramuscularly in three equal doses during the observation period. When mean oedema volumes were plotted the curves for Antistin, though separate, were much closer together than those shown in the salicylate experiment (Fig. 1), whilst the curves for Anthisan were practically superimposed. Thus, the result of the rabbit test of Antistin was confirmed and Anthisan was shown to have practically no effect at all upon Arthus reactions in guinea-pigs.

These results are surprising in view of the efficacy of Antistin and the inefficacy of sodium salicylates in the acute anaphylaxis experiments recorded above and they indicate that the action of sodium salicylate is not just a direct

anti-histamine effect. Furthermore they suggest that factors, other than histamine, are important in the development of the Arthus phenomenon.

The Shwartzman Reaction.

Effect of Sodium Salicylate.

The Shwartzman phenomenon was produced in rabbits as described by Shwartzman (1938). Berkefeld N filtrates were prepared from agar cultures of two strains of *Bact. coli* from the National Collection of Type Cultures. Injections of 0·25 ml. of *Bact. coli* 86 filtrate were made at two or three separate sites into the shaved abdominal skin and 24 hours later a dose of 0·1 ml. per kg. to 0·5 ml. per kg. of the less toxic *Bact. coli* 7020 filtrate was injected intravenously. Any areas of haemorrhage and oedema which developed were measured at 5 hours and 24 hours. In some experiments the treated animals received intraperitoneal injections of 3 per cent sodium salicylate at a dosage of 0·4 g. per kg. 15 min. before and 7½ hours after the intracutaneous injections, and a further two doses half hour before and 6½ hours after the intravenous injection. In other experiments the last two doses were omitted, salicylate being administered during the skin preparatory period only. Control animals received no treatment.

The results of seven such experiments are presented in Table V. Whilst there were no significant differences between treated and untreated groups as

TABLE V.—*Effect of Sodium Salicylate on the Shwartzman Reaction.*

Experiment.	Number of rabbits.	Treatment.	Areas of haemorrhage (cm²).			
1	2	Salicylates in Stages 1 and 2	0	0		
	2	Controls	0·64	1·75		
2	2	Salicylates in Stages 1 and 2	0·88	1·1		
	4	Controls	3·1	3·1	1·86	1·7
3	2	Salicylates in Stage 1	0	0		
	2	Controls	1·1	0·4		
4*	5	Salicylates in Stage 1	0	0	0	0
	5	Controls	0·4	0·1	0	0
5	3	Salicylates in Stage 1	0	0	0·1	
	3	Controls	2·8	0·8	0·8	
6	2	Salicylates in Stage 2	0	0·2		
	2	Controls	0·1	1·5		
7	2	Salicylates in Stage 2	0	1·2		
	2	Controls	0·6	3·1		

* Filtrate used in Experiment 4 was of very low potency owing to storage at 4° C.

regards the degree of prostration following the intravenous inoculations, the effect of salicylate treatment was strikingly manifest in the severity of the haemorrhagic skin lesions. In all experiments in which treated animals showed any reactions at all these were trivial compared to those in the controls and in three experiments the salicylate entirely suppressed their development. Moreover, it is clear that it is not necessary to give the drug throughout both stages. Given in Stage 1 only it appears to be every bit as effective as when given in both.

The effect of Anthisan.

Four rabbits were given intramuscular injections of Anthisan; 50 mg. at 5 min. before the skin preparatory injections, 50 mg. 5 hours after and 25 mg. 18 hours after. Two of the animals were given a further injection of 50 mg. 5 min. before the intravenous inoculation of *Bact. coli* filtrate. Two control animals received no treatment.

In contrast to the salicylate experiments no appreciable differences between treated and control rabbits could be detected in respect of the areas of haemorrhage which are the essential feature of the Shwartzman reaction although oedema when it occurred was less in the treated group. The whole group reacted with such remarkable uniformity that mean areas of haemorrhage were identical in five rabbits and only slightly larger in the sixth, which was one of the treated animals. These results, therefore, confirm the conclusion drawn from the Arthus experiments that the focus of action of sodium salicylate is different from that of the histamine antagonists.

The Mode of Action of Salicylates.

Inhibition of histamine lesions.

The importance of histamine release as a factor in the development of hypersensitivity reactions suggested the study of the effects of salicylate treatment on lesions produced by intracutaneous inoculations of histamine. It is unnecessary to record the experiments in detail because whilst the work was in progress a report of the anti-histamine effect of sodium salicylate was published by Swyer (1948) and our own work fully confirms his claim that administration of sodium salicylate inhibits the increased capillary permeability associated with histamine skin lesions. In early experiments we used intravenous injection of 5 per cent trypan blue given 5 to 10 min. before intracutaneous inoculation of freshly-diluted histamine acid phosphate and the rabbits were divided into salicylate treated and untreated control groups, the drug being administered intraperitoneally. Later we repeated Swyer's experiments more exactly by using pontamine blue as the vital dye and using each rabbit as its own control by testing it both before and after sodium salicylate had been given either intraperitoneally or intravenously. Plasma salicylate levels were obtained on specimens obtained just before the histamine inoculations. It was shown in preliminary experiments that the necessary interval of 30 to 45 min. between first and second tests on the same animal did not in itself influence the extent of " blueing " at histamine inoculation sites. All the experiments showed definite reduction of extravasation of dye when the blood plasma contained an adequate concentration of salicylate.

The possibility that this action of sodium salicylate might depend upon the formation of an inactive salicylate-histamine complex was investigated by testing a series of salicylate-histamine mixtures. These were made in isotonic phosphate buffer solution pH 7·5 and contained, per 0·2 ml. volume, 0·005 mg. histamine acid phosphate with 1, 0·33, 0·11, 0·03, 0·01 and 0 mg. sodium salicylate. Each of four rabbits received intracutaneous inoculations of each mixture, and also of similar doses of salicylate without histamine after an intravenous inoculation of pontamine blue solution. The results were extremely uniform, readings at 15, 30 and 120 min. showing blueing at all histamine sites without any appreciable effect of sodium salicylate at any dose. The inoculations of the salicylate doses without histamine caused no blueing at all.

Effect on capillary permeability in the Arthus and Shwartzman reactions.

The vital dye technique was used to study the effects of sodium salicylate and Anthisan upon capillary permeability at Arthus reaction sites in rabbits. Reversed passive Arthus reactions were produced as in previous experiments except that a smaller dose of antibody (0·12 mg. antibody N) was used for the intracutaneous injections. Treated rabbits were given sodium salicylate 400 mg. per kg. intraperitoneally or Anthisan 20 mg. per kg. intramuscularly at the same time as the sensitizing intravenous inoculation of antigen. Additional control animals received skin inoculations of antibody but no antigen. The vital dye was injected intravenously 15 min. before the skin inoculations of antibody in the form of 5 per cent solution of pontamine blue, 0·7 ml. per kg. dose. Estimates of intensity and measurements of areas of blueing were made at intervals.

The results are given in Table VI. Anthisan had no appreciable effect although adequacy of dose was proved by complete inhibition of blueing at sites inoculated with 0·01 mg. histamine acid phosphate in the same animals. This makes all the more interesting the striking inhibition by salicylate treatment. In these salicylate rabbits the extravasation of dye was no more than occurred in the additional controls as a result of inoculation trauma.

TABLE VI.—*The Effect of Sodium Salicylate and of Anthisan on Capillary Permeability During the Arthus Reaction.*

Number of rabbits.	Drug treatment.	Degree of " blueing " at skin inoculation sites.					
4	Sodium salicylate, 400 mg. per kg.	+	+	±	±	..	..
2	Anthisan, 20 mg. per kg.	+++	+++	..	..	..	..
6	*Nil*	+++	+++	+++	+++	+++	+++
5	*Nil**	+	+	+	±	±	..

* These additional controls received no antigen intravenously; hence no Arthus reactions occurred at sites of antibody injections. The slight blueing is attributable to inoculation trauma.

A preliminary experiment was carried out with another anti-histamine drug, Phenergan, because of a recent report that it is capable of suppressing the development of the Shwartzman phenomenon in rabbits (Vallery-Radot, Halpan and Reber, 1948). Phenergan is a synthetic compound derived from pheno-

thiazine and intramuscular doses of 20 mg. per kg. were found to render the rabbits very lethargic. On this dosage there was no evidence of any effect upon extravasation of dye at the Arthus reaction sites though the amount of oedema in two female animals seemed to be definitely reduced. Here again, therefore, the action of an anti-histamine drug appears to differ fundamentally from that of salicylate.

A similar effect of sodium salicylate upon capillary permeability during the first or preparatory stage of the Shwartzman reaction was demonstrated in two experiments. Rabbits received intravenous inoculations of pontamine blue solution 15 min. before duplicate intracutaneous inoculations of 0·25 ml. *Bact. coli* 86 filtrate. Treated animals had received 400 mg. per kg. sodium salicylate intraperitoneally 45 min. previously whilst controls were given corresponding volumes of saline. In controls the *Bact. coli* filtrate inoculations produced localized areas of intense blueing of the skin, but in treated rabbits the leakage of dye was trivial in comparison. The results are summarized in Table VII.

TABLE VII.—*Effect of Sodium Salicylate on Capillary Permeability when Administered During First Stage of the Shwartzman Reaction.*

Experiment number.	Rabbits. Number in Group.	Treatment.	Degree of blueing.*			
1	2	Sodium salicylate, 400 mg. per kg.	±	±	..	..
	4	*Nil*	++	++	+	+
2	2	Sodium salicylate, 400 mg. per kg.	±	−	..	..
	2	*Nil*	++	++	..	..

* Plus signs indicate intensity and extent of blueing at skin sites 2 hours after the intracutaneous inoculation of a *Bact. coli* filtrate.

DISCUSSION.

From the experimental results reported above it is clear that sodium salicylate in blood concentrations of the order of 50 mg. per 100 ml. is without appreciable effect upon the course of acute anaphylactic shock in guinea-pigs. It may, however, inhibit the development of the Shwartzman phenomenon in rabbits and of passive Arthus reactions in both rabbits and guinea-pigs. This claim is somewhat at variance with the work of Fischel (1947) who concluded that no clear effect of salicylates on the Arthus phenomenon could be demonstrated although he obtained slight inhibition in the first few hours with doses comparable with those used by us. The discrepancy probably rests on differences of technique, for in Fischel's experiments variability of rabbit reactivity was not eliminated by using each animal as its own control, a practice we consider advisable whenever the nature of experiment permits.

The activity of sodium salicylate in these hypersensitivity reactions is almost certainly due, in large measure, to its stabilizing effect upon capillary permeability, an effect first demonstrated by Swyer (1948) by experiments in which histamine,

inoculated locally, was used to provoke increased permeability of the capillaries of the skin. Swyer's results were readily confirmed by us, but our experiments lend no support to his suggestions that salicylates have anti-histamine properties and that their anti-rheumatic and anti-histamine actions may be causally related. It is more probable that sodium salicylate directly affects the walls of the capillaries though the mode of action remains obscure.

If this is the case the contrasting behaviours of sodium salicylate and the anti-histamine drugs in the various types of experiment recorded become explicable. Acute anaphylactic shock in the guinea-pig is generally considered to be due to histamine release at tissue sites of great histamine sensitivity. It is thus not surprising that anti-histamine drugs can afford complete protection against immediate shock although the protected animals may show ill effects some time later, suggesting that factors other than histamine are also involved in the reaction. Similar protection is not to be expected from sodium salicylate which is apparently capable of stabilizing the threshold of capillary permeability but which, so far as is known, has no direct neutralizing action on histamine. In reactions of the Arthus type and in the Shwartzman phenomenon the position is reversed ; sodium salicylate exerts a protective action whilst the anti-histamine drugs are impotent. The part played by histamine in such local hypersensitivity reactions is obscure. The slow development of erythema, oedema and necrosis over a period of several hours contrasts with both the dramatic suddenness of anaphylactic shock and the rapid development and rapid disappearance of the local reactions produced by single intracutaneous injections of histamine. The tissue toxin responsible, whatever its nature may be, appears to be either formed or released at the reaction site continuously over a prolonged period and the mechanism involves transport of reagents like antigens or antibodies to the site via the blood stream. In such circumstances capillary permeability becomes a most important factor, and the fact that lowered permeability thresholds at the reaction sites are an integral part of the Arthus and Shwartzman reactions is easy to demonstrate directly by the *intra-vitam* dye technique. Just how and when the capillary change is induced remains unknown, though in the Shwartzman reaction it appears to occur during the first, or skin preparatory, stage as a result of the local injection of bacterial filtrate ; hence in this latter reaction a high blood concentration of sodium salicylate during the first stage only is almost as effective as when it is maintained over the whole period of reaction.

It is not possible to assess at the present time the significance of these results in the major problem of the pathogenesis of rheumatic fever. They throw no light on the question of etiology, but if local hypersensitivity reactions caused by the interaction of streptococci, or streptococcal-tissue complexes, or auto-antigens, with homologous antibodies are at the root of some of the pathological changes which characterize the disease, then the palliative effects of salicylate therapy become readily explicable on the basis of the stabilization of capillary permeability. Furthermore, the necessity for long continued dosage is obvious. It is, of course, not suggested that this action of salicylates is their only pharmacological effect. Other actions, such as the compensated alkalosis due to the induced hyperpnoea demonstrated by Reid (1948), may also play a part although in our experiments we were unable to detect any significant change in the alkali reserve of the plasma of rabbits maintained on sodium salicylate throughout the periods of observation.

SUMMARY.

Sodium salicylate in blood concentrations of the order of 50 mg. per 100 ml. confers no appreciable protection against acute anaphylactic shock in guinea-pigs. With the same technique of shock production the anti-histamine drug, Antistin, was found to protect completely against the immediate fatal effects.

In passive Arthus reactions in both guinea-pigs and rabbits, sodium salicylate exerts a protective action whilst the anti-histamine drugs are relatively impotent. The same is true to an even greater extent in the case of the Shwartzman phenomenon.

This protective effect of sodium salicylate is not a direct anti-histamine effect but appears to be due to some action on the local blood capillaries. The increased capillary permeability which normally occurs in such hypersensitivity reactions is thus prevented.

The implications of these results in respect of rheumatic fever are briefly discussed.

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AN INVESTIGATION INTO THE EFFECT OF COMBINED DIPHTHERIA-TETANUS-PERTUSSIS PROPHYLACTIC.*

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WHEN different antigens are combined to give a polyvalent mixture, and the mixture intended for human prophylaxis, it is of particular importance to ascertain the effect of the presence of the others on the antigenicity of a given one.

It has been established that when some combinations of antigens are used, the response to one may suffer appreciably, compared with its activity as an isolated antigen. Michaelis (1904) called this phenomenon "The competition of antigens," and his opinion has been confirmed by Glenny and Waddington (1926), Hektoen and Delves (1932) and others. On the other hand, Tomcsik (1939), while studying this phenomenon, stressed the importance of the time factor, since the individual antibody responses from a combined preparation, may reach their maximal values at different times, and the duration of the immunity produced may also be different. In view, therefore, of the different responses, the best time to give the second inoculations may be difficult to judge accurately.

Reactions provoked by combined antigens constitute an entirely separate problem; we have shown, in respect of combined diphtheria-scarlet fever prophylactic, that there is a cumulative effect on repeated inoculation (Faragó, 1938).

This communication is concerned with the results of studies made in respect of the first two problems only.

METHODS.

The diphtheria toxoid used in these experiments was prepared by the method of Holt (1948); the purity of this material was 1,100 Lf/mg. N.

The *H. pertussis* vaccine was produced in the semi-synthetic medium of Cohen and Wheeler (1946) modified to give a solid medium by the addition of 2·5 per cent agar. The microbes derived from cultures on this medium were proved to be in Phase I.

The tetanus toxoid was prepared from culture in the semi-synthetic medium of Mueller and Miller (1941). The crude tetanus toxoid contained the equivalent of 3300 guinea-pig M.L.D./ml. of toxin. This was purified by the use of charcoal, and repeated adsorption onto $Al(OH)_3$ and dialysis: The final product had a purity of 39,000 eq. M.L.D./mg. N.

For these experiments we used two preparations: (i) A suspension of *H. pertussis* in a solution of tetanus and diphtheria toxoid, and (ii) an *adsorbed* combined prophylactic using Holt's $AlPO_4$ (1947) as the mineral carrier; this at a concentration of 7 mg. $AlPO_4$ ml.

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For *controls* we used the three components of the combined prophylactic separately, each with and without the addition of the mineral carrier. It should be recorded that all these preparations were preserved by the addition of 1/10,000 sodium ethyl mercuri thiosalicylate, and passed the usual sterility and innocuity tests.

Composition of the Reagents Employed.

(per ml.).

Type.	Combined.	Isolated.
"Fluid" (1)	10 Lf diphtheria toxoid.	(2) 10 Lf diphtheria toxoid.
	20.10^9 *H. pertussis*.	(3) 20.10^9 *H. pertussis*.
	100 eq. M.D.L. tetanus toxoid (2 Lf U.).	(4) 100 eq. M.L.D. tetanus toxoid (2 Lf U.).
Adsorbed (5)	10 Lf diphtheria toxoid.	(6) 10 Lf diphtheria toxoid with 7 mg. $AlPO_4$.
	20.10^9 *H. pertussis*.	(7) 20.10^9 *H. pertussis* with 7 mg. $AlPO_4$.
	100 eq. M.L.D. tetanus toxoid, 7 mg. $AlPO_4$.	(8) 100 eq. M.L.D. tetanus toxoid 7 mg. $AlPO_4$.

Groups of five chinchilla rabbits of the same breed, over 3 kg. in weight, were inoculated subcutaneously with 1 ml. of each of the eight preparations detailed above; the second injections were also 1 ml.

The antibody responses were determined at short intervals after inoculation, by taking ear-vein bleedings, and making accurately pooled samples of the sera from each group of animals. The diptheria antitoxin titrations were made using the method of Jensen (1933) against a standard antitoxin kindly supplied by the Statens Seruminstitut, København. Tetanus antitoxin titrations were made using a slight modification of Istratis (1938) micromethod, and *H. pertussis* agglutination titres with a heat-killed standard suspension of *H. pertussis*, also checked against a standard immune-serum.

RESULTS.

The results obtained from each preparation are shown graphically in Fig. 1–8.

1. *In respect of diphtheria toxoid*, we find that the responses to plain toxoid (non-adsorbed or mixed with vaccine) was the production of very small amounts of antitoxin, and this only reached a useful level after the third injection (Fig. 4). The same amount of toxoid adsorbed onto 7 mg. of $AlPO_4$ gave a greatly superior antitoxin response, in that even 21 days after one injection the serum titre was as high as after the third using plain toxoid, and the titre was still rising in respect of the adsorbed toxoid (Fig. 3). Even so, the best antitoxin responses were obtained after two or three doses of the combined prophylactic together with $AlPO_4$ (Fig. 1).

2. *In respect of tetanus toxoid*, the results obtained were, by and large, similar to that using diphtheria toxoid; using plain toxoid, a poor response followed the first dose, and sluggish responses to the second and third (Fig. 8). Combination of tetanus toxoid with diphtheria toxoid and *H. pertussis* vaccine gave much better results (Fig. 2), and also by adsorption onto $AlPO_4$ (Fig. 7), and the best results using the combined prophylactic with mineral carrier (Fig. 1). It may be pointed out here that the recovery from the negative phase following

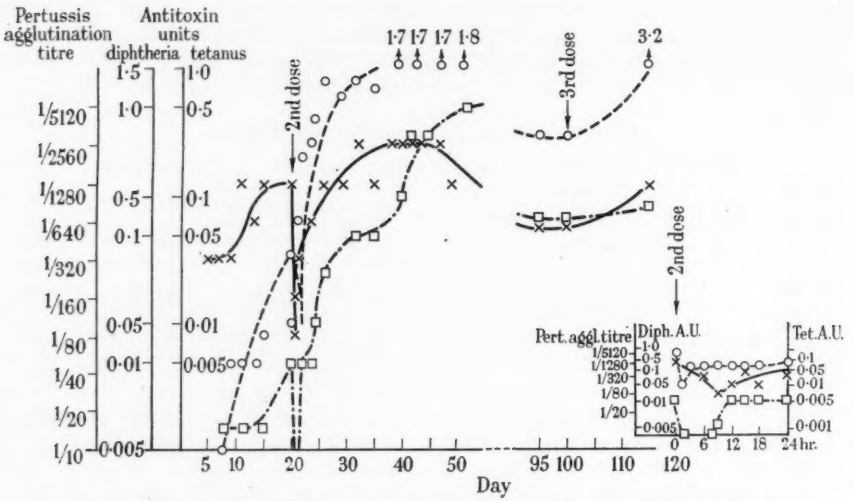

FIG. 1.—The combined diphtheria-pertussis-tetanus vaccine adsorbed to $AlPO_4$.
○— —○ Diphtheria. □ · — · □. Tetanus. ×———× Pertussis.

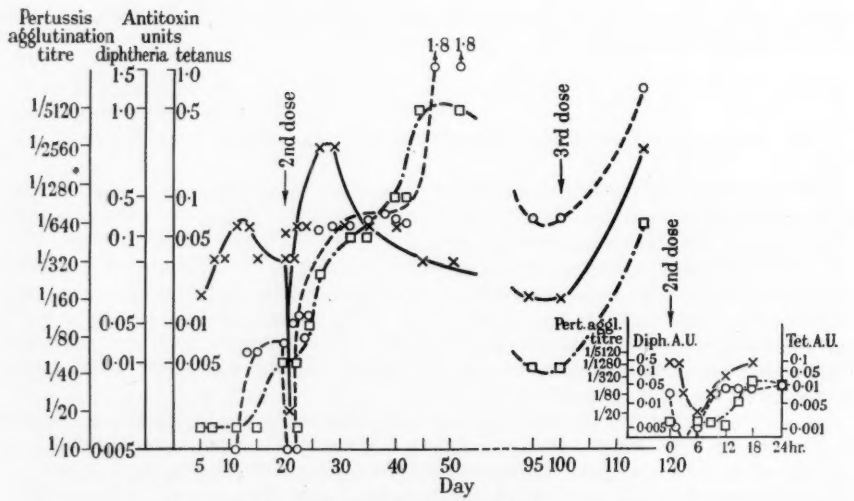

FIG. 2.—The combined diphtheria-pertussis-tetanus vaccine.
○— —○ Diphtheria. □ · — · □ Tetanus. ×———× Pertussis.

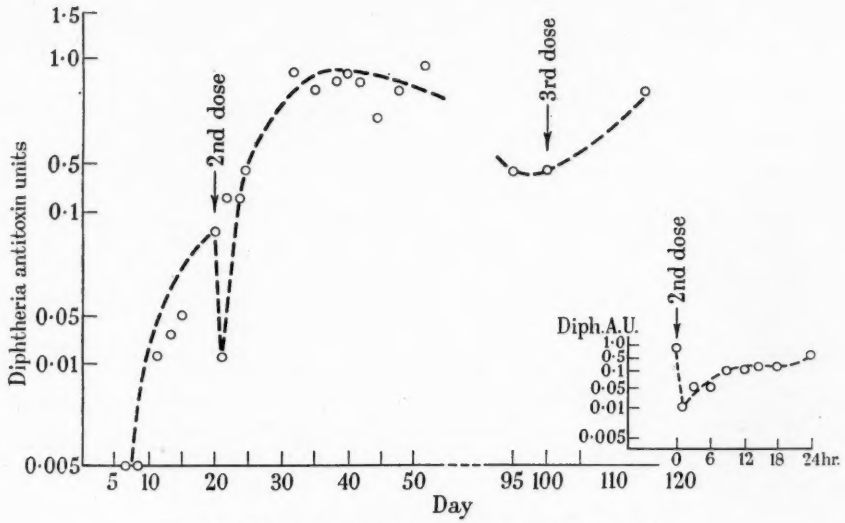

FIG. 3.—Diphtheria toxoid purified and adsorbed to $AlPO_4$.
○ — — ○ Diphtheria.

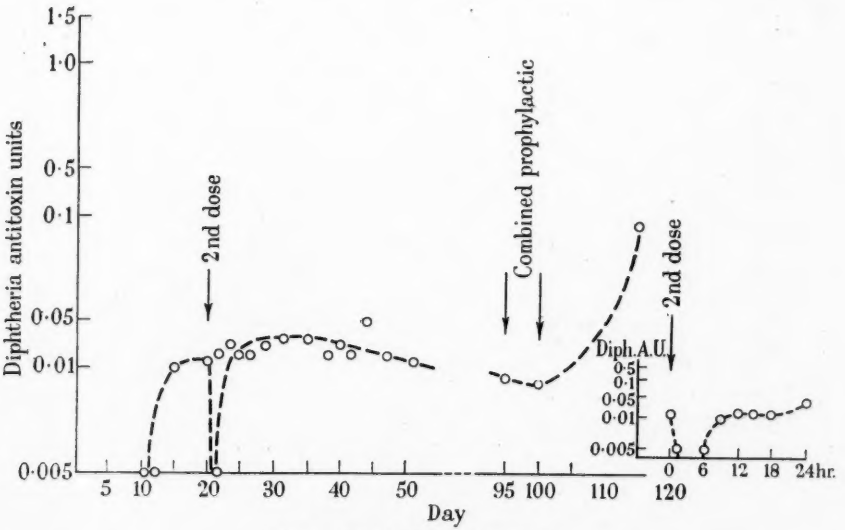

FIG. 4.—Diphtheria toxoid purified.
○ — — ○ Diphtheria.

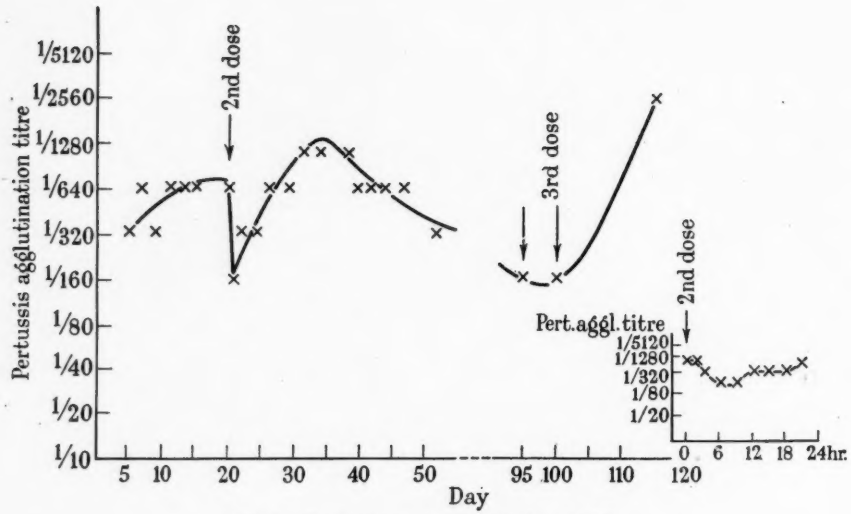

FIG. 5.—Pertussis vaccine adsorbed to $AlPO_4$.
× ——— × Pertussis.

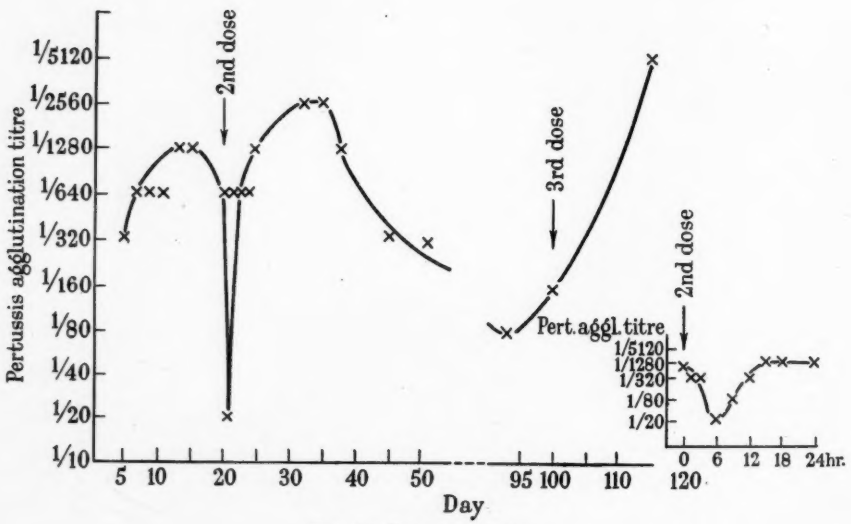

FIG. 6.—Pertussis vaccine.
× ——— × Pertussis.

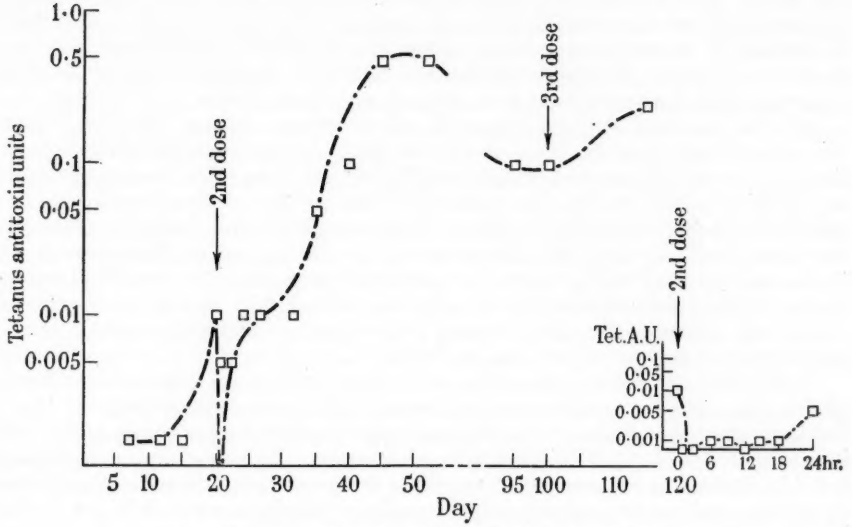

FIG. 7.—Tetanus toxoid purified and adsorbed to $AlPO_4$.
□ · — · □ Tetanus.

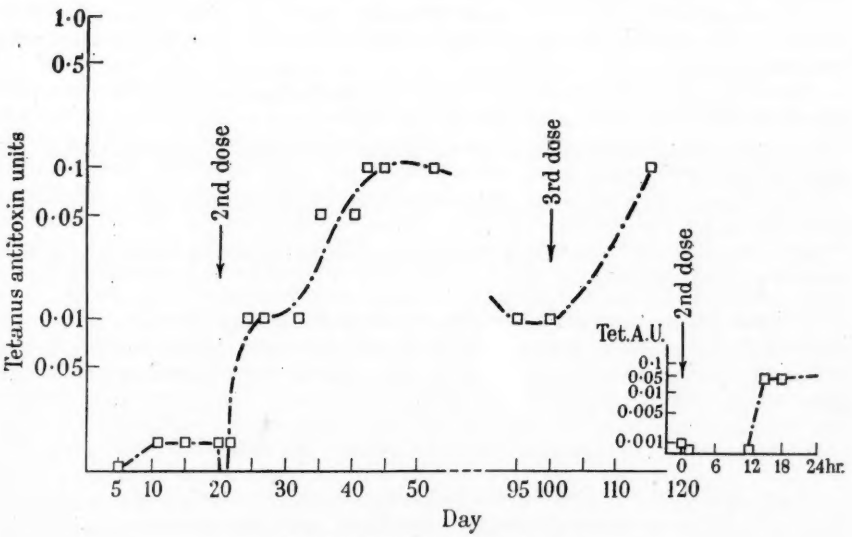

FIG. 8.—Tetanus toxoid purified.
□ · — · □ Tetanus.

the second injection was not as steep as with diphtheria antitoxin, but showed a marked lag before ascending to its peak value. This phenomenon was definite in respect of all three components using the combined preparations, and particularly noticeable in respect of tetanus antitoxin responses, and is probably a phenomenon directly referable to the negative phase.

3. *In respect of H. pertussis*, no marked differences were obtained. After the second and third inoculations of the plain vaccine there occurred a rapid rise and decrease in agglutination titre (Fig. 6). In respect of maximum titres, no significant differences were observed between the results from either the combined prophylactic (Fig. 1 and 2). or with mineral carrier (Fig. 5), all values were comparatively uniform. Nevertheless, it will be noticed that the response to the vaccine with $AlPO_4$ carrier, was definitely flatter, which suggests that the immunity induced was slower to become established, and slower to recede (Fig. 1 and 5); alternatively, the duration of antigenic stimulation may have been prolonged by association with the carrier.

Some points of interest also arise with regard to the negative phase. Details of this phenomenon are shown in the bottom right-hand corner of Fig. 1–8 where the time scale is in hours. Observations were made one hour before the second injection, 1, 3, 6, 9, 12, 16 and 18 hours afterwards. In every instance was there a fall in circulating antibody corresponding to the antigen injected, although less in degree, but more protracted in the case of antigens with mineral carrier. This latter may be due to the greater amount of antibody present in the animals before injection, and/or to a less ready fixation of the antigen by host antibody. The most marked decline in titre followed the second injection of tetanus toxoid, and this persisted for as long as twelve hours, less pronounced in respect of diphtheria antitoxin, and only slight in the case of pertussis agglutinins.

Certain considerations led us to determine to what extent, if any, the injection of non-specific substances had a stimulating effect on pre-existing antibody responses.

For this we employed two rabbits from each group; 75 days after their second inoculations, they were injected as follows:

(i) Those which received the $AlPO_4$ carrier with their former injections were given a dose of *E. typhi*.

(ii*a*) Those which received plain tetanus or diphtheria toxoid, a dose of *H. pertussis*, and

(ii*b*) Those which received *H. pertussis* vaccine alone, or tetanus toxoid, a dose of plain diphtheria toxoid.

Neither the non-specific, nor the non-related tertiary inocula caused any increase in the animals' pre-existing antibody titres, except, to a small degree, following the injection of typhoid vaccine. These results confirm our former observations (Faragó and Ujhelyi, 1942).

DISCUSSION.

From the point of view of the primary objective of this work, namely to determine if one or other of the three antigens used depressed the antigenicity of another when injected as a mixture, we have not observed any "competition of antigen" phenomenon. On the contrary, we have clear-cut evidence for

stating that the blending of *H. pertussis* vaccine with plain diphtheria and tetanus toxoids, increases markedly the antitoxin responses to the latter antigens.

Placing our findings in order of merit, the best results were obtained with the combined antigens associated with the $AlPO_4$ carrier, followed by the simple mixture, and the isolated toxoids a very poor third. In respect of the responses to the pertussis vaccine, it is to be noted that the agglutination titres obtained were substantially the same, no matter how the vaccine was inoculated.

As yet we do not know precisely the mechanism of the potency augmenting effect of the vaccine (pertussis) on toxoid antigens. It may possibly belong to the intricate pattern of depôt formation described by Faragó (1935), Holt (1947 ; 1949), and Holt and Bousfield (1949) ; on the other hand it may be due to other causes. We are at present investigating the latter possibility, and the results to date support the conclusions of Wagner-Sakharova (1937, 1938), Schütze (1940), Sauer and Tucker (1942) and Kendrick (1943). Ordmann and Grasset (1945) have recently described good results from a combined diphtheria-pertussis prophylactic using it for both human immunization and animal tests.

In respect of tetanus antitoxin production, in so far as the above experiments are referable to human prophylaxis, they would indicate that three spaced inoculations are advisable to ensure satisfactory immunization. The use of rabbits is perhaps to employ an animal that does not flatter antitoxin responses Maclean and Holt (1940), studying human material, found two doses of formol-toxoid without the addition of typhoid vaccine resulted in good immunity, and that the addition of the vaccine gave a five-fold increase in antitoxin response. Nevertheless, we recommend a greater quantity of tetanus toxoid in the combined prophylactic for human immunization.

In reference to the time factor in the responses to these antigens, as pointed out by Tomcsik, there is a very definite difference in the rates of production of diphtheria and tetanus antitoxins, and in agglutinins to *H. pertussis*.

These and other differences are most marked after the second inoculations. In respect of pertussis agglutinins, the time response curve of serum titres was always smooth ; they reached their maximum value after the second injection when combined with tetanus and diphtheria toxoids, some fifteen days when alone or associated with the $AlPO_4$ carrier, and twenty days when combined and associated with $AlPO_4$. The antitoxin response to diphtheria toxoid alone was as usual, giving a peak value ten days after the second injection, and some 17 days when adsorbed onto $AlPO_4$, but when combined with pertussis vaccine and tetanus toxoid showed a curious two-stage response, one reaching a maximum some 15 days after the second inoculation, and a second, some four times greater in amount, after 30 days, with a flat peak. Using the combined prophylactic associated with $AlPO_4$, the response was continuously smooth to give a maximum value at about the 20th day with a very flat peak, 12 days or more. In respect of the tetanus antitoxin responses, except perhaps in the case of plain toxoid, a two-stage response was obtained in every case, at approximately the 10th and 25th day after the second injection respectively. The significance of this phenomenon is obscure, but clearly one of importance when assessing potency, and in no way detracts from the value of such a combined prophylactic for human immunization.

Finally, our experiments dealt with the negative phase as one likely to be of some importance in practical immunization. The question seemed to be,

would the simultaneous inoculation of three antigens result in an exaggerated or prolonged negative phase ? A study of the figures detailing our results show that there is no essential difference using combined or isolated antigens, except that in the cases of the combined antigens plus $AlPO_4$, since the initial antibody titres were relatively high, the lowest titres obtained followed the use of isolated antigens. Madsen and Jensen (1943) who also used rabbits, analysed carefully the negative phase in respect of diphtheria antitoxin, and found it required three days for the height of the initial titre to return following injections of diphtheria toxin. This return to normal during the latent period for the second response phenomenon, would be accounted for by Glenny and Barr (1949) and Holt's contention (1949) that antitoxin is being continuously produced in an immunized animal. We are of the opinion that the less pronounced negative phase in our experiments, represent a state of affairs nearer to that obtaining in human immunization than that observed by Madsen and Jensen who used large amounts of toxin.

SUMMARY.

1. The comparative antigenicity of tetanus and diphtheria toxoids and *H. pertussis* vaccine were observed in combination ; and also adsorbed onto $AlPO_4$ gel, with the same antigens used alone, and adsorbed separately onto $AlPO_4$. Groups of five rabbits were used for each of the eight preparations, and the antibody titres determined on pooled sera.

2. The most effective preparation was the combined mixture with $AlPO_4$ carrier, followed by the combined mixture without mineral carrier. The simple unadsorbed toxoids gave very poor responses.

3. Although the presence of *H. pertussis* vaccine in the combined prophylactic resulted in a greatly enhanced antitoxin response from both the tetanus and diphtheria toxoids, there was no augmentation in the pertussis agglutinin titre from any mixture.

4. As the responses to primary, secondary and tertiary stimuli were closely followed, the different speeds at which antitoxic and antibacterial titres developed in the blood did not cause confusion in our reading of the results. Timing differences due to the use of $AlPO_4$ were thereby also accurately observed.

5. An apparently new and as yet unexplained phenomenon is described—a two-stage second response to tetanus toxoid, and under certain circumstances to diphtheria toxoid.

6. The negative phase, following reinoculation, was also measured. The immediate temporary fall in circulating antibody was most marked in the cases of plain toxoid, and less so with the $AlPO_4$ adsorbed toxoids. There was no exaggeration of the negative phase as a result of blending the three antigens.

7. The findings are discussed in relation to their possible mechanism and to human immunization.

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TISSUE NEUTRALIZATION OF LOUPING-ILL VIRUS.

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In an investigation of louping-ill in sheep, which had been inoculated subcutaneously with virus, it was frequently impossible to demonstrate virus in the brain after the animal had succumbed to encephalitis. In view of the observation of Gard (1944) that the brains of mice infected with poliomyelitis contained a neutralizing substance which appeared to be distinct from antibody, an examination has been made of the brains of sheep infected with louping-ill to ascertain whether failure to isolate virus was dependent on the presence of a similar neutralizing substance.

Examination of brains for demonstrable virus.

The brains of 30 sheep, in which fatal encephalitis had been induced by subcutaneous inoculation with louping-ill virus, followed three days later by an intracerebral injection of starch (Edward, 1947), were tested for virus shortly after the animals had died or had been killed when moribund. In only 19 (63 per cent) of the brains could virus be demonstrated by the intracerebral inoculation of mice. In 13 of these virus was plentiful, there being a 100 per cent mortality among the mice inoculated with a 10 per cent suspension. Titration of a few of the brain suspensions showed that the LD_{50} titre reached $10^{-4 \cdot 8}$. Virus was apparently scanty in 6 brains, because only a few of the inoculated mice died. In the other 11 (37 per cent) brains no virus could be demonstrated.

Among the strongly positive brains the earliest time at which the material was examined was the sixth day after inoculation with virus, and the latest was the ninth day. Two brains obtained on the thirteenth and fifteenth days were weakly positive. On the other hand, all but two of the brains found to be negative came from animals which succumbed between the tenth and sixteenth days. The exceptions were brains from two sheep in one experiment which showed nervous signs four days after inoculation and were seriously paralysed the next day. It is exceptional for louping-ill encephalitis to develop as soon as this, and, although the paralysis was typical, it is open to doubt whether the deaths were specific.

Of the sheep whose brains were examined, 6 had never been vaccinated against louping-ill; virus was present in all except one of these brains. The one negative brain was examined 14 days after inoculation, the animal having remained in a paralysed condition for 4 days. The other brains were obtained in challenge experiments from sheep which had succumbed in spite of attempts to protect them with formalinized vaccines. A few negative brains came from

animals vaccinated with materials whose inability to protect any of the animals inoculated suggested that they contained little or no specific antigen.

All except two of the brains found to contain virus were from sheep which had succumbed 24 hours or less after the first signs of encephalitis had been noticed. A higher proportion of those brains with no demonstrable virus came from animals which had persisted longer in a paralysed state. No virus, however, could be found in 6 brains even though they were examined 24 hours or less after encephalitis was first detected.

Neutralizing substance in infected brains.

In order to find out if the failure to demonstrate virus was due to the presence of a neutralizing substance, suspensions of the brains (10 per cent) were made, using as diluent M/50 phosphate buffer at pH 7·6 in 0·68 per cent saline. Mixtures of 1·8 ml. of brain suspension and 0·2 ml. of falling tenfold dilutions of virus, after standing for 3 hours at room temperature, were tested for virus by the intracerebral inoculation of mice. Suspensions of infected mouse or sheep brain provided a source of louping-ill virus. The LD_{50} titre was determined and compared with that of a similar control series of mixtures in a 10 per cent suspension of normal mouse brain. The presence of a neutralizing substance was deduced when the titre was significantly lowered by suspension in infected brain. The result was expressed as the neutralization index, which was the antilogarithm of the difference between the titres (expressed as the logarithms of the dilutions giving the 50 per cent mortality end-points) in the control and test mixtures. The infected brain suspensions were tested both unheated and after heating at 56° C. for 30 min. before adding the dilutions of virus, in order to destroy any living virus they already contained. It was thus possible to demonstrate a neutralizing substance in brains, even though they originally contained living virus.

Brain suspensions from 9 of 12 sheep neutralized virus to a significant degree, the titre of virus being reduced tenfold at least (Table I). Demonstrable virus was present in the 3 brains with no neutralizing activity, but was absent from 5 of those containing the neutralizing substance. One brain suspension had a neutralization index of 100; this animal, which had not received an injection of vaccine, had died 7 days after inoculation, the stage of paralysis having lasted 1 day. In spite of having the largest amount of neutralizing substance the brain suspension contained free virus. A suspension of normal sheep brain did not neutralize virus.

In two experiments no neutralizing substance was found in the pooled brain suspension of two paralysed mice, examined 8 and 9 days after intraperitoneal inoculation with louping-ill virus. Similarly the brain of a paralysed sheep, killed 6 days after infection by intracerebral inoculation, did not neutralize virus. Four suspensions, each made from the brains, spinal cords and spleens of 5 sheep, infected by the intracerebral route and killed 5 days later, had no neutralizing activity.

Tests for neutralizing activity were carried out on suspensions of spleen from three sheep, infected by peripheral inoculation, which had neutralizing substance in their brains. Two of the three suspensions, after being heated to 56° C. for 30 min., neutralized virus; before heating one of the positive suspensions contained free virus, but virus was not demonstrable in the other.

Properties of the neutralizing substance.

Brain suspensions were centrifuged at 3000 r.p.m. in an angle centrifuge for 2 hours and the supernatants were tested for neutralizing activity, allowing 3 hours' contact at room temperature with dilutions of virus. The supernatants appeared to neutralize virus to the same degree as the original suspension. Neutralizing activity was not affected by filtration of a suspension through a gradocol membrane of 800 mμ A.P.D. Although it withstood heating at 56° C. for 30 min., most of the neutralizing substance was inactivated at 70° C. for 30 min.; inactivation was complete at 100° C.

Serial dilutions of virus were inoculated intracerebrally into mice which 3 hours previously had received intraperitoneal injections of 1 ml. of brain suspension. No protection was afforded by brain suspensions which exhibited neutralizing activity *in vitro*.

Attempts were made to separate virus from neutralizing substances by the centrifugation of a neutral mixture. A series of dilutions of virus in suspensions of brain, after 3 hours' contact at room temperature, were centrifuged for 60 min. in the angle centrifuge and the supernatants were inoculated intracerebrally into mice. The end-point of the titration was the same as that of mixtures which had not been centrifuged.

To 13·5 ml. of brain suspension, 1·5 ml. of an infected mouse brain suspension, containing 600,000 M.L.D. of virus, was added, and the mixture was left for 3 hours at room temperature to allow combination of all the neutralizing substance with virus, there being excess virus present. The mixture was then heated at 56° C. for 30 min. to destroy virus, and afterwards tested for neutralizing activity by the method already described. As a control some of the original brain suspension without added virus was allowed to stand for 3 hours, similarly heated and then tested for neutralizing activity against dilutions of virus. Both the test and control mixtures effected the same degree of neutralization of virus. It appeared that heating had destroyed not only free virus, but also the virus which had combined with neutralizing substance, thus setting free the neutralizing substance in a fully active form, able to combine again with freshly added virus.

With 4 brain suspensions, which did not contain free demonstrable virus, it was possible to compare the neutralizing activity of materials inactivated at 56° C. with that of unheated materials; there was no significant difference. Since the neutralizing activity was not greater in the heated suspension, there was no evidence that in the fresh brain virus was present combined with neutralizing substance. The method, however, for estimating neutralizing activity was not sufficiently sensitive to detect the small amount of neutralizing substance capable of being liberated by heating if only a little combined virus had been present.

These findings suggested that the neutralizing substance was antibody. Although it was not possible to test the neutralizing substance against a wide range of viruses and thus demonstrate its specificity, it was shown that a brain suspension which neutralized louping-ill virus did not neutralize the virus of lymphocytic chorio-meningitis.

From 6 sheep, whose brains were examined, blood samples were taken just before death and the sera were tested for neutralizing antibodies to louping-ill

virus, using the method described by Edward (1948), except that buffered saline was employed as a diluent instead of serum-broth. All the sera contained antibody, but the amount was not proportional to the length of time (between 7 and 14 days) the animal had survived infection (Table I). Also there was

TABLE I.—*Results of Testing Brains for Neutralizing Activity.*

Sheep No.	Interval in days between inoculation and death.	Interval in days between onset of nervous signs and death.	Whether vaccinated.	Presence of virus in brain.	Neutralization index of heated brain suspension.	Neutralization index of serum.
S182	7	1	Not vaccinated	+	100	..
S154	11	1	Vaccinated	—	30	..
S183	6	1	Not vaccinated	+	30	..
49	14	4	,,	—	50	1000
21	12	1	Vaccinated	—	50	800
S157	17	11	,,	—	10	..
12	7	< 1	,,	+	10	30
37	8	1	,,	±	10	1500
29	10	2	,,	−	10	..
S185	8	1	Not vaccinated	+	< 10	..
16	7	< 1	Vaccinated	+	< 10	300
40	7	< 1	,,	+	< 10	60

no correspondence between the neutralizing activities of the serum and of the brain suspensions of a particular animal. Although in Table I the degrees of neutralization produced by both serum and brain suspensions are expressed as neutralization indexes, it must be pointed out that different techniques were used to estimate neutralization in the two materials.

DISCUSSION.

Gard (1944) described an inhibitory substance in the brains of mice, which had developed immunity to mouse poliomyelitis subsequent to infection. Mice were inoculated with a strain of virus of low virulence, causing myelitis only; 4 weeks later, when survivors were inoculated with the virulent encephalitic Theiler's FA strain, mice which had showed paralysis proved immune. After another 4 weeks their brains were shown to contain an inhibitory substance by titrating mixtures of brain suspension with dilutions of FA virus. The infectivity of the mixtures was less than that of similar dilutions of virus mixed with saline or normal mouse brain suspension. By centrifugation the greater part of the inhibitory activity was found in the deposit of brain tissue. If a mixture of virus and an inhibitory brain suspension was centrifuged in the angle centrifuge dissociation of virus and inhibitor took place, and the supernatant had an infectivity equal to that of the control mixture of virus and saline. The inhibitor was also effective *in vivo*; 0·5 ml. of brain suspension, injected 24 hours before or at the time of inoculation with virus, caused definite inhibition. Gard

concluded that the inhibitory factor was a substance residing in the infected and immune cell itself and was not antibody.

The neutralizing substance, found in the brains of sheep infected with louping-ill, differed from that described by Gard ; it would appear to be identical with neutralizing antibody. Although the neutralizing power of a brain suspension seemed to bear little relation to the level of serum antibody, it appeared that the chances of demonstrating virus in the brain after death were reduced the longer the animal survived after inoculation and the longer the period since the onset of encephalitis, both factors which would favour a high level of antibodies. Previous attempts at immunization, even though they failed to protect and were made with feebly antigenic material, also made the isolation of virus less likely.

It is well known that antibody in the tissues can make the isolation of virus impossible. The findings with louping-ill in sheep show that it may not be possible to isolate virus from the brain, even though it is examined within 24 hours after the first signs of encephalitis and only 10 days after injection of virus. They therefore emphasize the difficulties which may attend the diagnosis of neurotropic virus infections by isolation of virus from the brain.

SUMMARY.

Louping-ill virus could not be demonstrated in 11 out of 30 (37 per cent) brains, obtained from sheep which had succumbed to louping-ill after subcutaneous inoculation with virus.

A substance, capable of neutralizing virus *in vitro*, was found in the brains of some sheep infected by the subcutaneous route. It appeared to be identical with neutralizing antibody, and did not have the properties of a neutralizing substance, which had been described in the brains of mice infected with mouse poliomyelitis.

I wish to express my thanks to Mr. W. A. Fitzgerald for his assistance.

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